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(51) International Patent Classification ⁷ : C12N 15/79, 15/85, C12P 21/00, G01N 33/569, A61K 39/002, 38/17		A1	(11) International Publication Number: WO 00/46381 (43) International Publication Date: 10 August 2000 (10.08.00)
(21) International Application Number: PCT/US00/02966 (22) International Filing Date: 4 February 2000 (04.02.00) (30) Priority Data: 60/118,634 4 February 1999 (04.02.99) US 60/122,372 2 March 1999 (02.03.99) US 60/124,905 17 March 1999 (17.03.99) US 60/131,121 27 April 1999 (27.04.99) US (71) Applicant: UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; 632 Boyd Graduate Studies, Athens, GA 30602-7411 (US). (71)(72) Applicants and Inventors: GAERTIG, Jacek [PL/US]; Apt. #7, 134 Ashley Circle, Athens, GA 30605 (US). DICKERSON, Harry, W., Jr. [US/US]; 390 Hampton Court, Athens, GA 30605 (US). CLARK, Theodore, G. [US/US]; 206 Fairmount Avenue, Ithaca, NY 14850 (US). (74) Agent: SANDBERG, Victoria, A.; Muetting, Raasch & Gebhardt, P.A., P.O. Box 581415, Minneapolis, MN 55458-1415 (US).			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, I.K, I.R, LS, LT, LU, I.V, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: RECOMBINANT EXPRESSION OF HETEROLOGOUS NUCLEIC ACIDS IN PROTOZOA			
(57) Abstract <p>The ciliated protozoan <i>Tetrahymena</i> exemplifies a recombinant system for the expression of heterologous nucleic acids, preferably on the plasma membrane surface. Integration of a heterologous nucleic acid into the β-tubulin gene, <i>BTU1</i>, of a paclitaxel-sensitive <i>T. thermophila</i> mutant that possesses <i>btul-1K350M</i> β-tubulin allele allows screening for transformants using negative selection, as transformants have restored paclitaxel resistance. Transgenic ciliated protozoa of the invention can serve as live vaccines. For example, transgenic <i>Tetrahymena</i> expressing <i>Ichthyophthirius multifiliis</i> i-antigen protein on their surface are effective vehicles for vaccination of freshwater fish against infection by <i>I. multifiliis</i>.</p>			

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5 RECOMBINANT EXPRESSION OF HETEROLOGOUS NUCLEIC ACIDS IN PROTOZOA

This application claims the benefit of U.S. Provisional Application Serial No. 60/131,121, filed April 27, 1999; U.S. Provisional Application 60/118,634, filed February 4, 1999; U.S. Provisional Application 60/122,372, filed March 2, 1999; and U.S. Provisional Application 60/124,905, filed March 17, 1999, each of which is incorporated herein by reference in its entirety.

Statement of Government Rights

15 This invention was made with government support under grants from the National Institutes of Health (GM-54017-03) and the United States Department of Agriculture National Research Initiative Competitive Grants Program (NRICGP) (95-37204-2139). The U.S. government has certain rights in this invention.

Field of the Invention

The present invention relates to the field of recombinant protein production, particularly recombinant protein production in nonpathogenic protozoa, such as the ciliate *Tetrahymena*.

Background of the Invention

Efficient and high-level recombinant production of heterologous proteins is an important alternative to chemical synthesis and the isolation of proteins from native sources. Recombinant protein production is especially useful when the native protein is normally produced in limited amounts or by sources which are impossible, expensive and/or dangerous to obtain or propagate. Although a number of recombinant expression systems have proven useful for production of various heterologous proteins, none of these systems is universally applicable for

the production of all proteins. For instance, *E. coli* appears to lack the ability to provide many post-translational modifications to heterologous proteins. Yeast can provide only some post-translational modifications (e.g., glycosylation patterns), and rapid degradation of heterologous proteins in yeast is common.

- 5 Additionally, heterologous proteins secreted by yeast may contain long, untrimmed oligosaccharide chains, which sometimes results in biologically inactive or antigenically altered proteins. Moreover, a replacement of the natural mammalian signal peptide with a yeast signal peptide is almost always required for efficient secretion of mammalian proteins by yeast. Expression of
- 10 heterologous eukaryotic proteins in insect or mammalian cells can be more reliable but both require expensive media for cell propagation. Moreover, cultured insect cells and mammalian cells have a relatively long doubling time compared to conventional bacterial systems such as *E. coli* and certain protozoa such as *Tetrahymena*.

- 15 Protozoa represent an alternative for the recombinant production of heterologous proteins, however few protozoa have been characterized to the extent necessary for routine heterologous protein expression. Well-characterized pathogenic protozoa that have been genetically engineered to express heterologous proteins include *Trypanosoma cruzi*, *Trypanosoma brucei*, and
- 20 *Leishmania* spp. A number of shuttle vectors designed for episomal replication and coding region expression in pathogenic protozoa have been developed. An inducible coding region expression system has been established for pathogenic *T. brucei* (Wirtz, E., et al., *Science*, 268, 1179-1183 (1995)). Vectors that allow efficient coding region expression in different hosts like *E. coli* and mammalian
- 25 cells have also been developed (Al-Qahtani, A., et al., *Nucleic Acids Res.*, 24, 1173-1174 (1996)).

- Protozoa are characterized by a glycosylphosphatidylinositol (GPI) anchoring system that allows targeted surface expression, or "display," of various endogenous proteins. Recent experiments in the kinetoplastid
- 30 *Trypanosoma cruzi* demonstrated that mammalian and protozoan signal peptides function in *T. cruzi* to target a heterologous protein to different cellular compartments, and further showed both secretion and GPI-anchored surface

expression in *T. cruzi* of a heterologous protein (Garg et al., *J. Immunol.*, 158: 3293-3302 (1997)). Surface display in *T. cruzi* of chicken ovalbumin (OVA) was achieved using a construct comprising the signal sequence of *T. cruzi* glycoprotein, gp-72, that targets the protein to the endoplasmic reticulum, followed by a coding region for OVA, followed by 45 amino acids of amastigote surface protein I of *T. cruzi* which provided a C-terminal hydrophobic tail containing GPI anchor cleavage/attachment site. The protein thus anchored to the surface of the protozoan via a GPI structure was found to be readily presented in association with class I MHC by parasite-infected host cells.

Heterologous proteins have also been expressed in the slime mold *Dictyostelium discoideum*. A number of proteins have been expressed in this system including surface expression of the malaria circumsporozoite antigen (CSP) (Reymond et al., *J. Biol. Chem.* 1995, 270: 12941-12947); see Williams et al., *Current Biology*, 1995, 6:538-542, for a review).

Bioactive cytokines (IL-2 and IFN- γ) have also been produced in both *T. cruzi* and *Leishmania* (La Flamme et al., *Mol. Biochem. Parasitol.*, 75:25-31 (1995), and Tobin et al., *J. Immunol.*, 150:5059-5069 (1995)) in experiments that suggest that mammalian signal peptides are recognized and processed by these protozoa. However, pathogenic protozoa have not been exploited as a general purpose protein expression system, presumably because they are difficult or expensive to grow in large numbers and/or are infectious to human beings.

The nonpathogenic ciliate protozoan *Tetrahymena* has also been explored as a vehicle for expression of heterologous genes, but with limited success to date. *T. thermophila* has been successfully transformed using self-replicating palindromic ribosomal DNA (rDNA) purified from macronuclei (Tondravi et al., *Proc. Natl. Acad. Sci. USA* 83:4369 (1986)). Selection of transformants relied on a dominant paromomycin-resistance mutation in the 17S rRNA. rDNA-based shuttle vectors capable of autonomously replicating in *Tetrahymena* as well as in *E. coli* have also been developed; these plasmids contained a replication origin (ori) from the *T. thermophila* rDNA minichromosome (Yu et al., *Proc. Natl. Acad. Sci. USA* 86:8487-8491 (1989)).

rDNA vectors are usually circular vectors containing both regulatory regions and "coding" regions for *Tetrahymena* rRNA. A typical somatic rDNA vector contains a 5' nontranscribed sequence (5'-NTS), followed by a "coding" region for rRNA, followed by a 3' nontranscribed sequence (3'-NTS). A
5 transgene is inserted into the 3' NTS. Somatic rDNA vectors contain the macronuclear version of rDNA and transform either by replacement of the macronuclear rDNA gene via homologous recombination or by autonomous replication as an extrachromosomal element. Processing rDNA vectors, on the other hand, contain additional processing signals upstream and downstream from
10 the 5'-NTS and the 3'-NTS, respectively, obtained from the micronuclear version of rDNA. Processing rDNA vectors mimic what happens to the micronuclear rDNA in the newly developing macronucleus. After introduction of the vector into the developing new macronucleus during the sexual process of ciliates known as conjugation, the vector-borne micronuclear rDNA undergoes excision
15 and is maintained as an rDNA minichromosome (Yao et al., Mol. Cell. Biol. 9:1092 (1989)).

Both somatic and processing rDNA vectors have been used to insert a heterologous nucleic acid into a 3' nontranscribed spacer region of rDNA. For example, M.-C. Yao et al. (Proc. Nat'l. Acad. Sci. USA 88:9493-9497 (1991))
20 expressed cycloheximide resistance in *Tetrahymena* using an rDNA vector having the rpl29 cycloheximide resistant gene from *T. thermophila* inserted into the 3' nontranscribed spacer region (NTS) of the rDNA sequence. Similarly, P. Blomberg et al. expressed neomycin resistance in *T. thermophila* using an rDNA vector having the *neo* gene inserted into the 3' NTS, under control of rpl29
25 flanking sequences (Mol. Cell. Biol., 17:7237-7247 (1997)).

Gaertig et al. described an rDNA-based shuttle vector, *E. coli* vector pH4T2, that contains two replication origin (ori) fragments, followed by a 300 base pair 5' untranslated region obtained from the *HHF1* gene of *Tetrahymena*, followed by the prokaryotic gene for neomycin resistance, *neo*, followed by a 3'
30 untranslated region from *BTU2* from *Tetrahymena* (J. Gaertig et al., Nucleic Acids Res. 22:5391-5398 (1994)). Haddad et al. reported a small circular rDNA-based vector containing a repeat of the replication origin of rDNA (i.e., a

5' NTS), a *neo2* gene cassette (consisting of the *neo* gene under the control of histone *HHF1* promoter and the BTU2 transcription terminator) as a selectable marker, and a green fluorescent protein (GFP) cassette (also under control of *HHF1* promoter and BTU2 terminator) (A. Haddad et al., Proc. Nat'l. Acad. Sci. USA 94:10675-10680 (1997)). Rusconi et al. reported a circular vector containing the rDNA replication origin, *neo2* cassette, and a tRNA gene (Genes Dev. 10:2870-2880, 1996)).

A typical rDNA-based vector is a circular bacterial vector that contains a 5'NTS comprising two or more of ori sequences from *Tetrahymena* rDNA, followed by a selectable cassette marker such as the *neo 2* cassette (Gaertig et al., Nucleic. Acids Res. 22:5391-5398 (1994)). A nucleic acid fragment containing a heterologous coding region such as a transgene, flanked by a 5' untranslated region of a *Tetrahymena* gene (most often the ~30 bp 5' untranslated region of the *HHF1* gene of *Tetrahymena*) and a 3' untranslated region of a *Tetrahymena* gene (most often ~300 bp of the 3' untranslated region of the *Tetrahymena* gene *BTU2*), is typically inserted downstream of the selectable marker.

An rDNA construct that contains relatively short 5' and 3' untranslated sequences from two different protein coding genes of *Tetrahymena*, such as *HHF1* and *BTU2*, is unlikely to integrate into the *Tetrahymena* genome via homologous recombination at the corresponding protein-coding loci. It is more likely to insert into *Tetrahymena* rDNA as a result of a single crossover event which involves the replication origin fragment. In addition, an rDNA-based vector can be maintained as an extrachromosomal element; the ori from *Tetrahymena* rDNA is known to support extrachromosomal replication. The marker gene (e.g., *neo*), and the transgene, if present, are therefore most likely expressed from the transforming rDNA-based plasmid and/or as a result of insertion into genomic rDNA, and not by recombination with endogenous genes other than rDNA.

Due to frequent and unpredictable integration of sequences from rDNA vector and rDNA-based vectors into the native rDNA, however, levels of expression of recombinant gene products are presumed to be highly variable.

See J. Gaertig et al., Nucleic Acids Res. 22:5391-5398 (1994); R. W. Kahn et al., Proc. Natl. Acad. Sci. USA 90:9295-9299 (1993); W. J. Pan et al., Nucleic Acids Res. 23:1561-1569 (1995); and W. J. Pan et al., Mol. Cell Biol., 15:3372-3381 (1995). When relying on rDNA vectors for transformation, there is no way to

5 control the level of integration into the host chromosome, hence no way to control copy number and, as a result, the expression level of a heterologous protein. *Tetrahymena* contain about 45 copies of each protein coding gene in the macronucleus, and each copy contains about 10,000 pallindromic copies per macronucleus. Thus, using either of these types of vectors, it is possible for a

10 transgene to integrate at a similar copy number (10,000+). Overexpression of a transgene can be toxic to the protozoan host cell. Moreover, the loss of transgenes using these vectors cannot be prevented since this recombinant method generally lacks a reliable and sustainable means for selection. For example, a vector can contain both a transgene and a selectable marker, and both

15 may initially integrate into the protozoan host genome. However, subsequent cross-over events can eliminate the transgene while leaving the marker gene in the host genome, resulting in selection of cells that do not necessarily contain the transgene.

A protein expression system that provides for the efficient expression and

20 isolation of both prokaryotic and eukaryotic heterologous proteins in a nonpathogenic protozoan host is needed. In particular, a protein expression system that could provide surface expression of a heterologous prokaryotic or eukaryotic protein would constitute a much desired advance in the art.

25 Summary of the Invention

The invention provides a protein expression system that utilizes a protozoan for the production of eukaryotic and prokaryotic polypeptides, including proteins. In one aspect, the recombinant protein expression system of the invention includes a transgenic protozoan host cell that is resistant to

30 paclitaxel, wherein the host cell comprises a heterologous nucleic acid encoding a polypeptide. The recombinant protozoan host cell of this aspect of the invention is selectable by negative selection using paclitaxel. The protozoan

host cell that is transformed with the heterologous nucleic acid is preferably a *Tetrahymena* host cell containing a *btu1-1K350M* β -tubulin allele. In another aspect, the recombinant protein expression system of the invention includes a transgenic ciliated protozoan host cell that contains a heterologous protein
5 displayed on the plasma membrane surface of the host cell. Preferably, the surface-displayed heterologous protein is attached to the plasma membrane by a GPI anchor derived from an *Ichthyophthirius multifiliis* i-antigen.

Also provided by the invention is a novel protein expression vector. The vector contains a 5' flanking region followed by a heterologous nucleic acid
10 encoding a polypeptide comprising at least one targeting amino acid sequence encoded by a portion of an i-antigen-encoding nucleotide sequence from *I. multifiliis*, followed by a 3' flanking region. At least a portion of each of the 5' flanking region and the 3' flanking region is complementary to an endogenous gene of an intended host, so as to allow for integration into endogenous gene by
15 way of homologous recombination. In a preferred embodiment, the 5' flanking region and the 3' flanking region each contain a nucleic acid sequence selected from at least a portion of the *Tetrahymena* genes *HHF1*, *rpl29*, *BTU1*, *BTU2*, *SerH3* and the gene encoding actin.

The invention further provides a transgenic *Tetrahymena* that contains at
20 least a portion of an *I. multifiliis* i-antigen protein, preferably a portion of an *I. multifiliis* i-antigen protein that includes a targeting amino acid sequence.

Further, the invention provides a transgenic cell containing a heterologous protein that includes at least one targeting amino acid sequence encoded by an i-antigen-encoding nucleotide sequence from *I. multifiliis*. The
25 transgenic cell is not limited and can be a bacterial cell, a fungus cell, a protozoan cell, or an animal cell, for example. The targeting amino acid sequence is an N-terminal targeting sequence, a GPI cleavage/attachment sequence, or both. Preferably, the the heterologous protein is displayed on the surface of the plasma membrane of the transgenic cell.

30 The invention also provides a method for making a polyclonal antibody. An antigenic polypeptide is expressed on the surface of the plasma membrane of a transgenic protozoan host cell, then the transgenic protozoan host cell is

administered to an animal to generate an antibody response to the antigenic polypeptide. In another aspect of this embodiment of the invention, the antigenic polypeptide is cleaved from the surface of the host cell, isolated, then administered to an animal to generate an antibody response to the antigenic polypeptide. Optionally, the antibody is isolated from the animal.

The invention further provides a method for detecting antibodies to an antigenic polypeptide that involves expressing the antigenic polypeptide on the surface of a transgenic protozoan host cell; exposing the protozoan host cell to an antibody; and determining whether the protozoan host cell is immobilized.

10 Immobilization of the protozoan host cell is indicative of the presence of antibodies to the antigenic polypeptide. This method can be used to detect antibodies to a pathogenic parasite in the bodily fluid of a patient suspected of being infected with the parasite.

Also provided by the invention is a method for screening drugs for the ability to bind a polypeptide. Preferably, the drug to be screened is one that has cross-linking capability, but the drug screening method of the invention is not limited to just those drugs. The polypeptide is expressed on the surface of a transgenic protozoan host cell and the host cell is exposed to the drug. Binding of the drug is evidenced by an observable change in the swimming pattern of the host cell which may, but need not, included complete immobilization of the host cell. In the case of a drug having cross-linking capability, the drug is caused to cross-link. Cross-linking of the drug will generally immobilize the host cell, indicating that the drug has bound to the polypeptide.

The invention further provides a vaccine containing a transgenic nonpathogenic immunogenic protozoan which exhibits a surface-displayed antigenic polypeptide. The vaccine can be a live vaccine or a killed vaccine. In a preferred embodiment, the nonpathogenic immunogenic protozoan is *Tetrahymena* and the antigenic polypeptide includes at least an antigenic portion of an *I. multifiliis* i-antigen protein.

Also provided by the invention is a method for stimulating an immune response in a vertebrate that includes delivering to the vertebrate a transgenic

nonpathogenic immunogenic protozoan comprising a surface-displayed antigenic polypeptide.

The invention further includes a novel recombinant method for producing a polypeptide. A heterologous nucleic acid encoding the polypeptide is
5 introduced into a protozoan host cell, preferably a protozoan host cell that is sensitive to paclitaxel, to yield a transgenic protozoan host cell selectable by negative selection using paclitaxel. The polypeptide is then expressed in the transgenic protozoan host cell. Optionally, the polypeptide is displayed on the plasma membrane of the transgenic protozoan host cell and cleaved to release
10 the polypeptide. Also optionally, the polypeptide is isolated from the transgenic protozoan host cell. In another aspect, the recombinant method for producing a polypeptide includes introducing a heterologous nucleic acid encoding the polypeptide into a host cell to yield a transgenic host cell, wherein the polypeptide comprises at least one targeting amino acid sequence encoded by an
15 i-antigen-encoding nucleotide sequence from *I. multifiliis*, then expressing the polypeptide in the transgenic host cell.

Definitions

An "expression vector" is a nucleic acid molecule containing a nucleotide
20 sequence encoding a polypeptide that is capable of being expressed in a host cell. Generally, when the term "vector" or "vector construct" is used herein, an expression vector is intended. Typically, an expression vector is a DNA molecule that contains a gene, and expression of the gene is under the control of regulatory elements that can, but need not, include one or more constitutive or
25 inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene or other nucleic acid fragment is said to be "operably linked" to the regulatory elements.

A "cloning vector" is a nucleic acid molecule, typically a DNA molecule, that contains one or more restriction endonuclease recognition sites at which foreign nucleic acid fragments can be inserted in a determinable fashion without loss of an essential biological function of the vector, and often contain a
5 nucleotide sequence encoding a detectable and/or a selectable marker (i.e., "marker genes") that can be used to identify and/or select cells transformed with the cloning vector. Marker genes typically include nucleic acid fragments that encode polypeptides which can confer a phenotypic characteristic to the transformed cell, such as antibiotic resistance, test compound metabolism, and
10 the like.

Cloning and expression vectors can include naturally occurring or modified DNA or RNA, and can take the form of a plasmid, cosmid, or bacteriophage. Vectors can be linear or circular.

The terms "exogenous" or "heterologous," which are used
15 interchangeably herein, denote some item, typically a nucleic acid fragment or a protein, that is foreign to its surroundings. In particular, the terms apply to nucleic acid fragments that have been inserted into a host organism, but are not found in the normal genetic complement (i.e., genome) of the host organism. A nucleic acid fragment that is heterologous with respect to an organism into which
20 it has been inserted or transferred is sometimes referred to herein as a "transgene." A "transgenic" organism (whether microorganism or an animal) is a host organism that has been genetically engineered to contain exogenous (heterologous) nucleic acid fragments, including vectors. Introduction of the heterologous nucleic acids into a host cell to create a transgenic cell is not
25 limited to any particular mode of delivery, but includes, for example, microinjection, adsorption, electroporation, particle gun bombardment, liposome-mediated delivery and the use of viral and retroviral vectors.

Preferably, the heterologous nucleic acid fragments are stably integrated into the host genome, but they may, alternatively, be maintained
30 extrachromosomally. The heterologous nucleic acid fragments may but need not be inheritable.

A nucleic acid fragment is "excised" from genomic DNA by isolating genomic DNA from the host, as by cutting the nucleic acid fragment at one or more predetermined sites, for example at a restriction enzyme recognition site.

An excised nucleic acid fragment can be ligated into a vector and assayed
5 for recombination. Successful recombination can be detected, either directly or indirectly, as by using a laboratory assay or other detection procedure. Preferably, the presence or absence of a recombinant nucleic acid fragment is detectable by way of a chemical or biological assay. Detection can be mediated through the use of "reporter" nucleic acid fragments typically contained in the
10 vector, such as a particular nucleic acid fragment that can confer metabolic pathways for particular nutrient utilization or antibiotic resistance. For example, in a *lac* operon-based mutation detection system commonly used in *E. coli*, a mutation in a *lacI* mutation target gene affects the expression of the *lacZ* reporter gene, and expression of the reporter gene is detectable in an *E. coli* host by
15 assaying the ability of the host to produce the *lacZ* gene product (β -galactosidase) and thus metabolize a chromogenic substrate.

An "immunogenic" or "immune system stimulating" polypeptide, also referred to as a polypeptide antigen or an antigenic polypeptide, is one that
20 activates at least one cell type of the immune system of an animal, including phagocytic cells such as macrophages, as well as T cells and B cells. An example of an immune-stimulating ciliated protozoan is one that displays a polypeptide antigen on its cell surface such that an immune response to the antigen is elicited in an animal exposed to the immune-stimulating protozoan. Likewise, an immunogenic composition comprises an immunogenic polypeptide
25 and optionally contains a pharmaceutically acceptable buffer, such as phosphate buffered saline (PBS) or another buffer, recognized in the art as suitable and safe for introduction of proteins into a host to stimulate the immune system.

As used herein, the term "effective amount" refers to an amount of a substance that is effective to produce a desired result. For instance, an effective
30 amount of an immune-stimulating composition is one that is effective to activate cells of the immune system, including phagocytic cells such as macrophages, as well as T cells and B cells. The actual amount varies depending upon the health

and physical condition of a subject's immune system, i.e., to synthesize antibodies, the degree of protection desired, the formulation prepared and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

5 As used herein, the term "polypeptide" refers to a chain of amino acids linked through peptide bonds between an α -carboxyl carbon of one amino acid residue and the α -nitrogen of the next amino acid. The term polypeptide is used herein as a general term that includes polypeptides of any length, including what are commonly referred to in the art as peptides, oligopeptides, and proteins. A
10 "protein" contains one or more polypeptide chains that fold to adopt a particular conformation that has some type of biological activity or function. The properties and function of any particular protein are generally determined by the physical and chemical properties of the molecule. The order of the nucleotides in a nucleic acid fragment determines the order of amino acid residues in a
15 polypeptide and protein, i.e., the nucleic acid fragment "encodes" the polypeptide (protein).

Brief Description of the Drawings

Figure 1 is a schematic of the vector pBICH3.

20 Figure 2 shows full restriction maps of (a) *BTU1::neoI* construct (SEQ ID NO:1 and its complement, SEQ ID NO:2) and (b) pBICH3 vector construct (SEQ ID NO:3), as shown in as shown in Figure 1 (and its complement, SEQ ID NO:4); positions of restriction endonucleases sites are shown, with unique sites underlined; translation start and stop sites are boxed.

25 Figure 3 shows (a) an alignment of the deduced amino acid sequences of 48 kD (SEQ ID NO:5, upper line) and 55 kD (SEQ ID NO:6, lower line) i-antigens of *I. multifiliis*, where asterisks indicate identities between the two deduced protein sequences, double dots indicate highly homologous amino acids, and single dots indicate moderately homologous amino acids; boxes indicate
30 conserved regions; (b) an alignment of the nucleotide sequences of the coding regions of the *IAG48 [G1]* gene (SEQ ID NO:7, upper line) and the *IAG55 [G5]*

gene (SEQ ID NO: 8, lower line) of *I. multifiliis*, where asterisks indicate identities between the two nucleotide sequences.

Figure 4 is a schematic showing the experimental design used to introduce the *Ichthyophthirius* i-antigen into the *BTUI* locus of *T. thermophila*.

- 5 A hybrid gene composed of the *I. multifiliis* i-antigen coding sequence inserted between regulatory sequences of *BTUI* is integrated into the endogenous paclitaxel-sensitive *BTUI* locus by homologous recombination.

Figure 5 is a Western blot analysis identifying a putative 48 kD *I. multifiliis* i-antigen expressed by transgenic *T. thermophila*.

- 10 Figure 6A-6D are photomicrographs using immunofluorescence for detection of surface expression of a putative 48 kD *I. multifiliis* i-antigen in transgenic *T. thermophila*.

- Figure 7 shows Western blots of (a) total protein extracts of transformant cells; and (b) total culture medium from which transformed cells were removed, probed using anti- *I. multifiliis* i-antigen antibodies; the load of the medium shown in (b) is about 1/30th of the load of the cells shown in (a).
- 15

Figure 8 shows a Western blot of a total protein extract from *Tetrahymena* transformed with chicken ovalbumin, probed with anti-chicken ovalbumin antibodies.

- 20 Figure 9 is Western blot showing expression and secretion from a transformed *Tetrahymena* of a G1 i-antigen with C-terminal truncation.

Figure 10 shows ELISA results for fish vaccinated with *Tetrahymena* expressing (a) membrane associated G1 i-antigen and (b) secretory form of G1 i-antigen.

- 25 Figure 11 is a 10X magnification of serotype A *I. multifiliis* an immobilization test conducted using sera (1:20 dilution) from (a) fish vaccinated with live *Tetrahymena* expressing *neo* ("anti-live Tneo," the negative control) and (b) fish vaccinated with live *Tetrahymena* expressing the full-length 48 kD i-antigen protein from a G1 *I. multifiliis* isolate ("anti-live TG1").

Detailed Description

The invention provides a protein expression system that utilizes a protozoan, preferably a nonpathogenic ciliate, more preferably *Tetrahymena*, for the production of eukaryotic and prokaryotic polypeptides, including proteins.

- 5 The protein expression system of the invention is useful to produce virtually any polypeptide for any purpose. It is suited to both large scale and analytical scale production of recombinant polypeptides, and is particularly useful for expression of polypeptides that are difficult to produce in conventional recombinant protein expression systems.

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Ciliated protozoa

- Protozoa, particularly nonpathogenic protozoa, are well-suited for use as recombinant protein expression systems. Not only are many protozoa capable of secreting proteins, but they often contain an anchoring system (known as a GPI anchor) that allows surface expression, or "display," of various endogenous proteins without secretion. Some protozoa, like *Tetrahymena*, have major membrane proteins that are GPI-anchored; they in essence contain a GPI-anchored coat of surface-displayed proteins on the plasma membrane. These displayed proteins can often be cleaved from the cell surface of a protozoan by phospholipase C (typically isolated from *B. thuringiensis*). Many nonpathogenic protozoa are fast-growing and inexpensive to culture. Nonpathogenic ciliated protozoa can be environmentally friendly if the desired genetic modifications are performed within their somatic genomes localized to the macronucleus; heterologous nucleic acids that have integrated into the macronucleus, such as those that disrupt the *BTU1* gene in *Tetrahymena*, are lost when the protozoan undergoes the sexual process of conjugation. The old macronucleus disintegrates, and the new macronucleus is formed as a result of differentiation of the zygotic micronucleus. As a result, a nucleic acid that had integrated into the macronucleus is not inherited or otherwise perpetuated, a feature which enhances environmental safety by preventing heterologous nucleic acids from entering the gene pool.
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Protozoa are also more effective than yeast in providing post-translational modifications to certain eukaryotic proteins. For example, yeast apparently lacks a number of post-translational modifications typically found on tubulins, such as acetylation of Lys40 in α -tubulin, polyglutamylation and polyglycylation of α - and β -tubulin (T. MacRae et al., Eur. J. Biochem. 244:265-278 (1997)). On the other hand, these tubulin modifications were found in *Tetrahymena* and other ciliates like *Paramecium* (J. Gaertig et al., J. Cell Biol. 129:1301-1310 (1995)); V. Redeker et al., Science 266:1688-1691 (1994)). Ciliates, in particular, are excellent candidates for heterologous polypeptide expression; the presence of cilia vastly increases cell surface area, providing more opportunity for expression of GPI-anchored polypeptides.

A transgenic nonpathogenic ciliated protozoan of the invention is preferably a free-living ciliate; that is, it can propagate without a host. Examples of nonpathogenic free-living ciliates include *Tetrahymena*, *Paramecium*, *Blepharisma*, *Colpidium*, *Euplotes*, *Stylonichia* and *Oxytricha*. More preferably, the free-living transgenic nonpathogenic ciliate is *Tetrahymena*. *Tetrahymena* can be grown in large volume cultures using a variety of inexpensive media including skimmed milk powder. Generation time is short (1.5-3 hr) and cells attain remarkably high density given their size (~50 μ m in length). Indeed, it has been reported that fermentation in perfused bioreactors has permitted growth to concentrations as high as 2.2×10^7 cells/ml, equivalent to a dry weight of 48 g/l. See, e.g., T. Kiy et al., Appl. Microbiol. & Biotech., 38:141-146 (1992). Further, *Tetrahymena* has the ability to secrete proteins into the growth medium, and mutant strains defective in the release of hydrolytic enzymes have been isolated (P. Hunseler et al., Dev. Genet., 13:167-173 (1992)). Mutant strains lacking or exhibiting reduced levels of secreted hydrolytic proteases are preferred for surface expression or secretion of a heterologous polypeptide that is sensitive to proteolysis by hydrolytic enzymes. Finally, a large part of the cell metabolism is devoted to the production of abundant surface membrane proteins known as immobilization antigens (i-antigens), whose expression is tightly regulated by environmental conditions. This feature makes *Tetrahymena* a preferred host because expression of a heterologous protein containing an endogenous GPI

anchor can potentially be controlled by changes in temperature. J.R. Preer, The Molecular Biology of Ciliated Protozoa, pp. 301-339 (ed. J.G. Gall), Academic Press, New York (1986).

- Tetrahymena*, which has genomic DNA generally on the order of 75% A-T, offers additional advantages as a host system for the cloning and expression of genes from other organisms with AT-rich genomes. For example, a number of human pathogens (for example, malarial plasmodia, mycoplasmas, etc.) have extremely AT-rich genomes. Genomic DNA contains four bases (A, T, C, G), and DNA duplexes typically hybridize by means of A-T and G-C base pairing.
- In the genomes of many organisms, the amounts of A, T, C, and G are relatively equal, thus their genomic DNA contains roughly the same number of A-T and G-C base pairs. "AT-rich" genomes or nucleic acids are genomes or nucleic acids that have an AT content of more than about 50% of the total bases in the fragment, preferably about 65% or more, and more preferably about 70% or more. Notably, AT-rich fragments are inherently unstable in conventional systems such as *E. coli*, making cloning genes from AT-rich organisms into conventional systems difficult. As an AT-rich host, however, *T. thermophila* is expected to stably maintain heterologous nucleic acid fragments having "AT rich" sequences.
- Tetrahymena*, like other ciliates, utilizes UAA and UAG as codons for the amino acid glutamine, while most other organisms recognize those as termination codons. Thus, *Tetrahymena* is particularly useful to express heterologous genes that are derived from other ciliates. Expression of ciliate genes in conventional (eukaryotic and prokaryotic) protein expression systems often requires that the ciliate gene be mutated so as to utilize the conventional nucleic acid code in order to produce a full-length protein.

Furthermore, immobilization of *Tetrahymena* in culture with specific antibodies offers a potential bioassay for detection of specific antibodies in biological samples that would shorten the time and lower the cost for screening, and would obviate the need for chemical detection substrates used in more standard diagnostic tests.

Transformation of the protozoan host with a heterologous nucleic acid

- The protozoan host is transformed with a heterologous nucleic acid, which can be either integrated into the host's genome or maintained extrachromosomally on an autonomous plasmid or other construct. The
- 5 heterologous nucleic acid encodes a polypeptide that is expressed by the resulting transgenic host. There is no limitation on the type of heterologous polypeptide that can be expressed in the protozoan host. The polypeptide coding region of the vector construct can, for example, be a coding region derived from a pathogenic protozoan, or from another eukaryotic or prokaryotic organism. As
- 10 already noted, a protozoan host such as *Tetrahymena* is especially useful as a vehicle for creating an expression library for organisms with AT-rich genomes, particularly pathogenic organisms, such as *Plasmodium* (the protozoan agent of malaria). Genomic fragments from these organisms are difficult to clone and stably maintain in *E. coli*. Another example of a preferred heterologous nucleic
- 15 acid sequence is one that encodes at least a portion of an antigenic polypeptide in that is capable of stimulating an immune response in an animal upon exposure to the polypeptide. In a particularly preferred embodiment of the invention, a transgenic ciliated protozoan displays an antigenic polypeptide on its cell surface.
- 20 The location of the expressed polypeptide is determined by the protein targeting sequences, if any, encoded by the heterologous nucleic acid and thereby incorporated into the resulting polypeptide. The heterologous polypeptide can accumulate in the cytosol, can be secreted from the host cell, or can be embedded in or anchored to the host cell plasma membrane, as is further described below.
- 25 Preferably, the heterologous nucleic acid is introduced into the ciliate host on an expression vector that is capable of integrating into the host's genome. In a particularly preferred embodiment of the invention, expression vectors operate by way of homologous recombination with a highly expressed gene that is endogenous to the protozoan host, such as a β -tubulin gene as
- 30 described below. On the other hand, an expression vector that does not rely on the regulatory sequences of an endogenous gene for expression can, optionally, be maintained extrachromosomally in the ciliated protozoan host cell. An

expression vector maintained as an extrachromosomal element is preferably an rDNA-based vector containing ori from *Tetrahymena* rDNA, which is known to support extrachromosomal replication. Such a vector further includes a 5' regulatory region from an endogenous *Tetrahymena* gene containing a promoter region operably linked to the heterologous coding region and, optionally, a 3' regulatory region from the same or, preferably, a different *Tetrahymena* gene. For example, expression vectors can contain regulatory regions from ciliate genes such as *HHF1*, *rpl29*, *BTU1*, *BTU2*, *SerH3*, and those encoding actin.

10 *Expression vectors*

Expression vectors useful for transforming protozoa in accordance with the invention can be generally classified into three types: replacement vectors, rDNA vectors, and rDNA-based vectors. Replacement vectors accomplish DNA-mediated transformation by replacing or altering endogenous genes using homologous recombination. Integration of the heterologous nucleic acid into the host's genome at the targeted site is accomplished via homologous recombination involving a double crossover event with the vector containing the heterologous nucleic acid. An example of an expression vector useful for genomic incorporation of a heterologous nucleic acid by replacement is one that includes a heterologous coding sequence flanked by portions of the endogenous *BTU1* gene of *Tetrahymena*.

A replacement vector thus preferably includes a 5' region, followed by a heterologous coding region, followed by a 3' region, wherein at least a portion of each of the 5' and 3' regions is complementary to 5' and 3' regions on an endogenous gene of the host, to allow for genomic integration of the heterologous coding region via homologous recombination. The 5' and 3' regions of the vector optionally contain regulatory elements, such as a promoter and a terminator; alternatively, the necessary regulatory elements are supplied by the endogenous gene into which the heterologous coding region integrates. Preferably, the 5' and 3' regions of an expression vector useful in *Tetrahymena* include sequences complementary to the *BTU1*, *BTU2*, *SerH3* or *HHF1* genes of *Tetrahymena*. Optionally, a replacement vector further includes a nucleotide

sequence encoding a selectable marker, such as *neo*. Preferably the marker sequence is under the control of its own promoter, thus another regulatory region is included 5' to the sequence encoding the selectable marker; and example is the *neo2* cassette.

5 β -Tubulin is a cytosolic protein in *Tetrahymena* that is a monomeric constituent of cytoskeletal elements known as microtubules. An α/β tubulin dimer is the building block of microtubules, and both α -tubulin and β -tubulin are essential proteins. *T. thermophila* expresses two major β -tubulin genes, *BTU1* and *BTU2*, which encode identical β -tubulin proteins (J. Gaertig et al., Cell Mot. Cytoskel., 25:245-253 (1993)). It was determined that either of these two genes (but not both at once) can be disrupted without a detectable change in the cell phenotype.

 Substitution of lysine (K) 350 by methionine (M) in the *BTU1* gene to yield the *Btul-1K350M* allele confers increased resistance to several
15 microtubule-depolymerizing drugs (oryzalin, colchicine, vinblastin), and increased sensitivity to a microtubule-stabilizing agent, paclitaxel (J. Gaertig et al., Proc. Nat'l. Acad. Sci. USA 91:4549-4553 (1994)). The phenotype of the *Btul-1K350M* allele is expressed even in the presence of wild-type copies of the second β -tubulin gene, *BTU2*. Cells carrying the *btul-1K350M* allele can be
20 transformed to paclitaxel resistance by gene replacement of *btul-1K350M* with a wild-type *BTU1* gene fragment, thus eliminating the need to incorporate a means for positive selection. Because the *BTU1* gene is not essential for survival, any loss-of-function mutation of *btul-1K350M* in the presence of wild-type *BTU2* gene confers paclitaxel resistance.

25 It has been discovered as part of this invention that heterologous nucleic acids can be inserted into a β -tubulin gene of *T. thermophila* for successful cell-surface expression that is advantageously maintained by way of negative selection. Expression of a heterologous protein in *T. thermophila* is preferably accomplished using a transgenic *T. thermophila* that contains the negatively
30 selectable *Btul-1K350M* allele of the β -tubulin gene (*BTU1*) to direct heterologous nucleic acid fragments to this highly expressed locus by

homologous recombination. A transformed cell line can thus be readily identified by paclitaxel selection, in that successful transformation restores paclitaxel resistance.

Accordingly, a preferred expression vector in accordance with the present invention preferably includes a nucleic acid fragment capable of integration into either the *BTU1* or *BTU2* gene of *T. thermophila*, preferably the *BTU1* gene, more preferably the *Btu1-1K350M* allele of the *BTU1* gene. The *BTU1* gene contains 5' and 3' regulatory regions that flank the coding region; hence the preferred expression vector contains at least a portion of each of these regulatory regions, which portions are sufficient to allow for homologous recombination with the endogenous gene. The flanking sequences of *BTU1* that are included in the vector (e.g., Figs. 1 and 2) allow for targeting of the heterologous coding region into the endogenous *BTU1* gene via homologous recombination with the endogenous gene.

The protozoan host to be transformed with the vector is preferably *T. thermophila* which contains a β -tubulin gene (*BTU1*) wherein lysine 350 is substituted by methionine (*btu1-1K350M*). The expression vector disrupts the *btu1-1K350M* gene by homologous recombination-mediated insertion of a heterologous nucleic acid, thereby restoring resistance to paclitaxel in the resulting transgenic host. Homologous recombination can take place at sites within the coding region of the highly expressed target gene or at sites that flank the gene target, which include but are not limited to flanking regulatory regions; all that is required is that the expression of the target gene be disrupted. When *BTU1* is the target gene, a second β -tubulin gene (*BTU2*) remains available to provide the essential function of β -tubulin *in vivo*, such that *BTU1* can be fully replaced with *btu1-1K350M* (or heterologous nucleic acid sequence) without adverse effects to the organism. When *T. thermophila* having the *btu1-1K350M* allele of the *BTU1* gene is used as the host, transformants can be maintained by negative selection, since transformed mutants are not sensitive to paclitaxel while nontransformed mutants are. Transformants are thus readily identified by negative selection by growth on in media containing paclitaxel; there is no need to engineer drug-resistance or other means of selection into the genome of the

transgenic host. Additionally, there is no limitation on the nature of the heterologous nucleic acid sequence that can be targeted to the *BTUI* locus of *T. thermophila*.

The heterologous nucleic acid sequence that is incorporated into the host genome in accordance with the present invention optionally encodes a selectable marker. For example, an expression vector can include a *BTUI* derivative, *BTUI-2::neol* (see Fig. 2) which substitutes the coding region of a prokaryotic gene, *neol*, for that of *BTUI*, to provide a means for positive selection of transformants. The *neol* gene confers resistance to paromomycin in *T. thermophila*, and a *BTUI-2::neol* fragment can then be used to generate *BTUI* gene knockouts by homologous recombination and positive selection with paromomycin. Of course, when the host organism is a *T. thermophila* mutant containing the *btu1-1K350M* allele of *BTUI*, transformants are identified with negative selection and a positive selection is not necessary.

Optionally, a replacement vector for use in *Tetrahymena* includes a temperature-regulatable promoter region to facilitate controlled expression of the heterologous coding region. The temperature-regulatable promoter can be supplied instead of or in addition to another promoter, such as the promoter region of the endogenous *BTUI* gene. The temperature-regulatable promoter is preferably positioned within the 5' untranslated region of the vector, more preferably between a 5' BTU region and the heterologous coding region. Because *Tetrahymena SerH3* encodes an i-antigen whose expression is temperature dependent, the 5' regulatory region of the *SerH3* gene of *Tetrahymena* may include a temperature-regulatable promoter that is useful in the invention.

Also optionally, the replacement vector contains a second promoter region, in addition to the promoter region supplied by the endogenous target gene, for enhanced expression of the heterologous protein.

In a representative example of the invention, the expression vector (pBICH3, Fig. 1) includes the entire coding sequence of the i-antigen gene inserted in a correct translational frame between the regulatory sequences of the *BTUI* gene. This vector contains a complete plasmid sequence commercially

available under the trade designation pBLUESCRIPT SK (+), from Stratagene, La Jolla, CA, and is generally useful for integration of any heterologous coding region into the β -tubulin gene of *T. thermophilis*. One skilled in the art will recognize that other plasmids may be equally suitable, such as those

- 5 commercially available under the trade designation pCRScript, from Stratagene, La Jolla, CA; pGEM from Promega, Madison, WI; and pCRII, from Invitrogen, Carlsbad, CA.

Figure 2(b) shows the full restriction map of pBICH3, represented by SEQ ID NOs:3 and 4. The complete nucleic acid sequence (SEQ ID NO:7) and
10 deduced amino acid sequence (SEQ ID NO:5) of the DNA encoding the 48 kD *Ich* i-antigen is presented in Figure 3. Nucleic acid sequence SEQ ID NO:7 of Figure 3(b) is included in pBICH3 at base pair 1000 to base pair 2325 as shown in Figure 2(b). The fragment GCAAGCTTG at base pair 991 to 999, which follows the start codon ATG and immediately precedes the i-antigen coding
15 sequence, is a cloning residual from the parent construct, HHF1::neo (R. Kahn et al., Proc. Nat'l. Acad. Sci. USA 90:9295-9299 (1993)). The resulting vector construct can be propagated in *E. coli* as a shuttle vector as well.

Gene regulatory elements useful in such transformation systems include both upstream (5') and downstream (3') regulatory elements. For example, in
20 prokaryotic and eukaryotic genes, the upstream region contain promoter elements that specify correct initiation of mRNA synthesis. Although classical promoters have not yet been identified in ciliates, examination of the upstream regions of various ciliate genes shows the presence of sequence elements that are highly conserved, and which are known to be required for gene expression in a
25 wide variety of other eucaryotic systems. For example, TATA sequences that play a critical role in transcription initiation in most eukaryotes, are present in the 5' flanking regions of a number of ciliate genes (for example, Cupples et al. (1986) Proc. Natl. Acad. Sci. USA 83:5160; Brunk et al. (1990) Nucl. Acids Res. 18:323; Tondravi et al. (1990) Mol. Cell. Biol. 10:6091; Prat et al. (1986) J. Mol. Biol. 189:47). Less common eucaryotic promoter elements (consisting of
30 CCAAT sequences) are also present. The upstream regions of ciliate genes contain information that is both necessary and sufficient for promoter function.

Because promoter elements are subject to differential regulation and have profound effects on RNA transcription levels *in vivo*, different promoters can be used for the expression of the heterologous nucleic acid. These include the promoters from the actin, histone H4 and *SerH3* i-antigen genes from

5 *Tetrahymena thermophila* (Cupples et al. (1986) Proc. Natl. Acad. Sci. USA 83:5160; Brunk et al. (1990) Nucl. Acids Res. 18:323; and Tondravi et al. (1990) Mol. Cell. Biol. 10:6091). Expression from the actin promoter is typically constitutive, while expression from the histone and *SerH3* promoters are cell-cycle and temperature-dependent, respectively. Expression vectors capable of

10 autonomous replication must include a promoter element; however, when the heterologous nucleic acid is genomically integrated, inclusion of a heterologous promoter element in the construct is optional, as the 5' regulatory region of the endogenous gene can be utilized instead.

The sequences required for correct 3' processing of mRNA transcripts in

15 ciliates (that is, transcription termination and polyadenylation) lie in close proximity to (and approximately 100 bp on either side of) the 3' terminus of most ciliate RNA transcripts. For example, an additional region from the 3' end of the *SerH3* i-antigen gene from *T. thermophila* can be added to the heterologous nucleic acid prior to cloning and expression.

20 rDNA vectors and rDNA-based vectors, including the typical components of each, were discussed in detail in the background. Although not preferred embodiments of the invention, these vectors can nevertheless be effectively used to express heterologous proteins in *Tetrahymena*. Depending upon the vector design, rDNA vectors and rDNA-based vectors can integrate

25 into the host genome, for example via homologous recombination or a single cross-over event, or they can be maintained extrachromosomally. Either type of vector can be constructed to include a heterologous protein containing an N-terminal targeting sequence, a C-terminal targeting sequence (i.e., a GPI anchor) or both, for targeted expression of a heterologous protein in *Tetrahymena* and

30 other ciliates. Preferably, the N-terminal targeting sequence and/or the C-terminal targeting sequence is derived from an i-antigen of *I. multifiliis*.

Targeted protein production

Advantageously, the invention allows the recombinant production of a heterologous polypeptide to be targeted within or outside of a cell. The targeting mechanism is not limited to protozoan hosts but can be used in any type of cell, for example a mammalian cell, a yeast cell, a protozoan cell, or a bacterial cell. Preferably, however, the cell is a cell of a ciliated protozoan. Optionally, therefore, a recombinant genetic construct of the invention (i.e., expression vector or transgenic organism) contains a heterologous nucleic acid that encodes a protein comprising an N-terminal protein targeting sequence or a C-terminal protein targeting sequence, or both. An N-terminal targeting sequence directs a protein to the endoplasmic reticulum (ER), and as a result can cause the protein to embed in the plasma membrane or to be secreted out of the cell. A C-terminal targeting sequence, specifically a GPI anchor, can cause the protein to be displayed on the membrane surface of the host cell. These targeting sequences can be either endogenous to the host cell, as where the heterologous nucleic acid is introduced into the host's genome by way of homologous recombination with a native coding sequence, or heterologous to the host cell.

Thus, to direct a heterologous polypeptide to the plasma membrane surface ("surface display"), the vector construct preferably encodes a heterologous polypeptide comprising (1) an amino terminal signal peptide and (2) a GPI cleavage/attachment sequence (i.e., a "GPI anchor"). The expression system of the invention is thus uniquely suited for expression of membrane proteins such as receptor proteins or proteins that utilize a GPI anchor. In other applications in which secretion of a heterologous polypeptide is intended, the vector construct preferably encodes a heterologous polypeptide comprising an amino terminal signal peptide but not a GPI cleavage/attachment sequence. Finally, for intracellular production of the heterologous polypeptide, the heterologous polypeptide encoded by the vector construct preferably includes neither an amino terminal signal peptide nor a GPI cleavage/attachment sequence.

Surprisingly, surface display of heterologous proteins in *Tetrahymena* is not limited to the use of a GPI anchor modification domain that is native to

Tetrahymena. It has been discovered, for example, that proteins that contain a GPI anchor derived from an immobilization antigen ("i-antigen") of *Ichthyophthirius multifiliis* are displayed on the membrane surface of *Tetrahymena* (Example II). Likewise, it is not necessary to use N-terminal signal peptides derived from *Tetrahymena*; it is shown in Example VI that the N-terminal signal peptides of both *I. multifiliis* i-antigen and *T. cruzi* gp-72 protein work well in *Tetrahymena*.

Thus, protein targeting sequences can be derived, for example, from *I. multifiliis*, *T. cruzi*, *Paramecium*, *Dictyostelium*, *Leishmania*, *Toxoplasma*, *Crithidia*, *Plasmodium* and *Chlamydomonas*. An example of an N-terminal protein targeting sequence from *T. cruzi* is the N-terminal signal peptide of the gp-72 surface protein. Heterologous protein targeting sequence obtained from *I. multifiliis* are described in T. Clark et al., Proc. Nat. Acad. Sci. USA, 89:6363-6367 (1992) and T. Clark et al., Gene 229:91-100 (1999); additional exemplary N-terminal and C-terminal targeting sequences, derived from the 48 kD and 55 kD *I. multifiliis* i-antigens (SEQ ID NOs:5 and 6, Fig. 3(a)), are described in provisional U.S. patent application Serial Number 09/124,905, filed March 17, 1999. The coding sequence of an *I. multifiliis* i-antigen includes two putative signal elements. For example, the N-terminal portions (i.e., about the first 20 amino acids) of the 48 kD i-antigen (SEQ ID NO:5, Fig. 3(a)) and the 55 kD i-antigen (SEQ ID NO:6, Fig. 3(a)) form highly hydrophobic domains which are similar to the signal peptides required in other organisms for translocation of newly translated polypeptides into the endoplasmic reticulum (ER). The C-terminal portions (i.e., about the last 20 to 23 amino acids) of the 48 kD i-antigen (SEQ ID NO:5, Fig. 3(a)) and the 55 kD i-antigen (SEQ ID NO:6, Fig. 3(a)) contain sequences that are homologous to the signals required for addition of a glycosylphosphatidylinositol (GPI) anchor and membrane anchoring of surface-displayed polypeptides. Surprisingly, the targeting signals of the *I. multifiliis* 48 kD i-antigen have been discovered according to the invention to be fully functional in *Tetrahymena*, causing surface display on the *Tetrahymena* plasma membrane of the i-antigen of *I. multifiliis*. Heterologous fusion proteins comprising one or both of these *I. multifiliis* signal sequences are therefore fully

expected to be targeted correctly in *Tetrahymena*. For example, the targeting sequences of the *I. multifiliis* i-antigen can be used for surface display in *Tetrahymena* of heterologous surface membrane proteins such as surface antigens or receptors. Moreover, these targeting sequences are also expected to
5 allow surface display or secretion of a polypeptide that is not normally expressed on a cell surface. For example, the protein expression system of the invention can be used for surface display of proteins that may be toxic to the cell when expressed intracellularly, such as proteases that can degrade *Tetrahymena*'s own essential cytosolic proteins.

10 It should be understood that this aspect of the invention involving protein targeting using, for example, N-terminal and C-terminal targeting sequences from *I. multifiliis* and other protozoa applies not just to protein targeting in protozoa but in other cells such as bacterial cells, fungus cells and animal cells including vertebrate cells such as mammalian cells.

15 Further, other types of targeting sequences are well-known and can be used in the practice of the invention. For example, targeting to the surface of a ciliate can also be achieved if the protein includes a known transmembrane domain, such as the C-terminal 80 amino acids of human membrane cofactor, in place of a GPI anchor (see e.g., J. Seeber et al., *J. Cell Sci.*, 111:23-29 (1998)).

20 In this embodiment, the protein can be partially embedded in the membrane and partially exposed on the outside of the cell.

It should also be understood that recombinant genetic constructs of the invention encode proteins that preferably do, but need not, contain N-terminal and/or C-terminal targeting sequences; a protein without such signaling
25 sequences will simply remain in the cytosol of the host cell.

Cell surface expression of antigens

The ability to express polypeptides on a protein surface, in particular, gives this system advantages over others known in the art for many purposes.

30 For example, antigens, including haptens, can be expressed on the cell surface as fusion proteins, cleaved, isolated, and injected into laboratory animals to generate antibodies for further use. Alternatively, antigens expressed on the

surface of a host cell can be used to identify antibodies directly using a *in vitro* immobilization reaction or chemokinesis of swimming cells. Antisera directed against the i-antigens of hymenostomatid ciliates (including *Paramecium*, *Tetrahymena* and *Ichthyophthirius* spp.) cross-link cilia and cause rapid

5 immobilization of corresponding cell types in culture (J. Preer, "Surface Antigens of *Paramecium*" in J. Gall, ed., Molecular Biology of Ciliated Protozoans, Academic Press, London, 301-339 (1986); F. Caron et al., Ann. Rev. Microbiol. 43:23-42 (1989); Clark et al., Ann. Rev. Fish Dis. 5:113-131 (1995)). For example, an immobilization assay can be conducted on the transgenic cells

10 to detect antibody in serum samples of a parasite-infected patient. A positive reaction is detected by immobilization of the live transgenic protozoan cells, due to cross-linking of antibodies bound to surface-displayed parasitic proteins. This method can be extended to detect serum antibodies to other bacterial, parasitic, or fungal infections or diseases, using a live transgenic protozoan genetically

15 engineered in accordance with the invention to display the appropriate antigen on its surface. Likewise transgenic cells of the invention that display antigens on their surface can be used to isolate and purify antibodies. Screening of chemical agents (drugs) for the ability to bind to polypeptides, and purification of drugs thus identified, can also be readily accomplished using the invention, as long as

20 the candidate drugs possess cross-linking capability. Cross-linking capabilities include the provision of multiple potential binding sites on the candidate drugs, or the addition of chemical or radiation-dependent cross-linking sites on the candidate drugs. In the latter case, cross-linking is initiated after binding of the drug to the live ciliate, and the mechanism for cross-linking must be selected

25 such that it does not itself cause immobilization of the transgenic ciliate. For example, the protein expression system could be used for screening multivalent ligands that bind to human neurotransmitter receptors expressed on the surface of transgenic *Tetrahymena* according to the invention.

Preparative protein production

The invention includes a method for producing a polypeptide that involves introducing into a ciliated protozoan host an expression vector containing a heterologous nucleotide sequence encoding a polypeptide, then
5 expressing the polypeptide. After the vector is introduced into the ciliate host, the host is cultured under conditions that allow expression of the coding region such that the polypeptide encoded by the coding region is produced. The polypeptide can be present within the host cell, secreted from the host cell or is anchored to the surface of the host cell. Optionally, the method further includes
10 isolating the polypeptide. The amino acid sequence of the heterologous polypeptide encoded by the coding region of the vector used to transform the ciliate host optionally includes protein targeting sequences. The polypeptide produced by the transformed host preferably includes either an amino terminal signal peptide or a GPI cleavage/attachment site or both. More preferably, the
15 polypeptide includes both an amino terminal signal peptide and a GPI cleavage/attachment site.

Transgenic ciliate

The present invention also provides a ciliated protozoan, preferably a
20 nonpathogenic ciliated protozoan, more preferably *Tetrahymena*, that has been genetically engineered to express a heterologous polypeptide, preferably on its cell surface. A surface-expressed heterologous protein is attached by way of a "GPI anchor" that is encoded by a GPI cleavage/attachment sequence. Preferably, the ciliate contains a vector containing a coding region that encodes
25 the heterologous protein, wherein the coding region is integrated into the *BTUI* gene of *T. thermophila*.

Live and killed vaccines

Success in expressing polypeptides on the surface of a ciliated protozoan
30 has immediate implications for vaccine development. Surface antigens are important targets of the humoral immune response against a wide range of microbial pathogens. Accordingly, the present invention provides a system for

vaccination that makes use of surface expression of heterologous antigenic polypeptides encoded by heterologous nucleic acid fragments in a ciliated protozoan.

A vaccine effective for the prevention of infection in an organism is one
5 that elicits the production of a protective immune response in an organism exposed to the vaccine. The immune response can be a cellular response and/or involve the production of antibodies. The goal of vaccination is to elicit a population of lymphocytes, which upon subsequent exposure to the disease causing agent, such as a parasite, proliferate and produce antibodies and/or
10 effector cells specific to the parasite, resulting in protection against lethal infections.

The vaccine of the invention can be either a live vaccine or a killed vaccine. It is useful to prevent disease in vertebrates, including humans, dogs, cats, reptiles, poultry, cattle, swine and fish. Preferably, the vaccine of the
15 invention is effective to prevent disease in fish, more preferably it is effective to prevent parasitic infection in fish. The vaccine preferably comprises a live or killed host ciliated protozoan having, attached to its plasma membrane and displayed on its surface, a heterologous antigenic polypeptide. The heterologous protein is preferably GPI-anchored to the plasma membrane. In a particularly
20 preferred embodiment of the vaccine, the heterologous antigenic polypeptide that is surface expressed comprises all or an immunogenic portion of an immobilization antigen ("i-antigen") of a pathogenic ciliated protozoan. I-antigens are surface protein that are generally abundant on the surface of a ciliate; an antibody that is specific for an i-antigen causes a loss of protozoan
25 motility, and can cause agglutination of the cilia. Use of a whole cell vaccine, whether live or killed, has the additional advantage of potentially serving as an adjuvant, thus further stimulating the immune system of the recipient.

White spot disease, also called "Ich," is a disease caused by the parasite *I. multifiliis*, a holotrichous ciliated protozoan which is an obligate parasite of
30 freshwater fish. Under conditions of intensive aquaculture, Ich frequently has a high morbidity and mortality, resulting in significant financial losses to fish producers. Attempts to control or prevent *I. multifiliis* infections have met with

only limited success. Killed *I. multifiliis* cells and *I. multifiliis* cilia alone do not elicit protective immunity. Nor has vaccination of fish with the cells or cilia of other ciliates, such as *T. thermophila* cells or cilia, generated protective immunity against *Ich* infection (Gratzek et al., U.S. Pat. No. 4,309,416).

5 Chemical treatments are likewise unsatisfactory because carcinogenicity and/or toxicity are typically associated with these therapeutic agents. See Burkart et al., *J. Fish Dis.*, 13:401-410 (1990), for a summary. Moreover, *I. multifiliis* can only be grown in association with its host, and efforts to produce i-antigens in conventional protein expression systems are inconvenient at best.

10 Significantly, *Tetrahymena* is nonpathogenic and is normally present in freshwater ecosystems. It thus represents an ideal vehicle for vaccination against *I. multifiliis* infection and other fish diseases. The use of *T. thermophila* for the expression and delivery of the antigens will make possible large-scale, cost-effective fish vaccination programs.

15 A vaccine against *I. multifiliis* infection preferably comprises live or killed *T. thermophila* that have been engineered according to the invention to express all or an immunogenic portion of an i-antigen from *I. multifiliis* on its surface. Preferably, the i-antigen used in the live or killed *T. thermophila* vaccine of the invention is encoded by i-antigen encoding sequences of either
20 *LAG48[G1]* or *LAG55[G5]* (Fig. 3(b)). In a particularly preferred embodiment, the vaccine comprises transgenic *T. thermophila* wherein expression of the *I. multifiliis* i-antigen is achieved by insertion of the i-antigen into the endogenous *btu1-1K350M* locus. Because this transformant can be maintained by negative selection, a selectable drug-resistance gene does not need to be introduced into
25 the transgenic host, eliminating the possible introduction of antimicrobial resistance genes into the environment upon release of transgenic *Tetrahymena*.

In the ciliated protozoan genetic code, TAA and TAG encode glutamine, whereas they serve as stop codons in the "universal" genetic code. The presence of glutamine-encoding TAA and TAG codons in the native coding sequence of
30 the i-antigen creates barriers to expression of this sequence in conventional bacterial gene expression systems. Advantageously, *T. thermophila* recognizes UAA and UAG codons as glutamine codons as does *I. multifiliis*. In addition,

post-translational modifications, particularly glycosylation, are expected to occur more normally in organisms related to *Ichthyophthirius* (for example, *Tetrahymena*) than in prokaryotes such as *E. coli*, or more distantly related eukaryotes. Because such modifications can play a critical role in immune
5 recognition, *Tetrahymena* is particularly advantageous in accordance with the present invention.

As shown in the following Examples describing the surface localization of *Ichthyophthirius* proteins in *Tetrahymena* LAG48[G1] transformants, the N-terminal and C-terminal protein targeting peptides associated with *I. multifiliis* i-
10 antigen function appropriately in *Tetrahymena*, i.e., the antigenic protein is expressed on the surface of transgenic *Tetrahymena*. Moreover, the overall distribution of *I. multifiliis* antigens in transformed *Tetrahymena* cells bear a striking resemblance to the pattern seen in the parasite itself where they are found in ciliary and plasma membrane, and in the cell cortex in association with
15 secretory mucocysts.

The invention further includes a method for vaccinating an animal that includes contacting the animal with a genetically engineered nonpathogenic ciliated protozoan, preferably *Tetrahymena*, so as to elicit a protective immune response in the animal to the heterologous antigen displayed on the surface of the
20 protozoan host. For example, an animal can be exposed to an ingestible composition, such as food, water, or both, that contains an immunogenic genetically engineered nonpathogenic ciliate of the invention. Alternatively, an animal can be immersed in or sprayed with an aqueous solution that includes the immunogenic ciliate. For example, fish can be vaccinated in accordance with
25 the invention by exposing the fish to a composition including transgenic *T. thermophila* expressing an antigenic polypeptide (e.g., the *I. multifiliis* i-antigen to vaccinate against white spot disease). The fish can be immersed in an aqueous solution comprising transgenic *T. thermophila* expressing the *I. multifiliis* i-antigen. Because the antigenic proteins are presented to the immune
30 system, probably through the lateral line and gills, the fish can begin to produce protective antibodies to at least a portion of the *I. multifiliis* i-antigen.

The vaccine can also be administered by injecting the animal with live or killed genetically engineered nonpathogenic immunogenic ciliated protozoa, by injecting the animal with a purified or partially purified membrane fraction of cells of the genetically engineered nonpathogenic immunogenic ciliated protozoa, or by injecting the animal with cilia from cells of the genetically engineered nonpathogenic immunogenic ciliated protozoa. Additionally, genetically engineered protozoa that display an antigen on the surface according to the present invention can also be used to generate monoclonal antibodies directed to the surface-expressed polypeptide.

10

EXAMPLES

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

15 The examples utilize many techniques well-known and accessible to those skilled in the arts of molecular biology, and in the transformation of *Tetrahymena*. Enzymes are obtained from commercial sources and are used according to the vendors' recommendations or other variations known in the art. Reagents, buffers and culture conditions are also known to the art. References
20 containing standard molecular biological procedures include Sambrook et al. (1989) Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory Press, Plainview, New York; Maniatis et al. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Wu (ed.) (1979) Methods Enzymol. 68; Wu et al. (eds.) (1983) Methods Enzymol. 100 and 101;
25 Grossman and Moldave (eds.) (1980) Methods Enzymol. 65; Miller (ed.) (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Old and Primrose (1981); Principles of Gene Manipulation, University of California Press, Berkeley, California; Schlieff and Wensink (1982) Practical Methods in Molecular Biology; Glover (ed.) (1985)
30 "DNA Cloning", Vols. I and II, IRL Press, Oxford, UK; Hames and Higgins (eds.) (1985) Nucleic Acid Hybridization, IRL Press, Oxford, UK; Setlow and

Hollaender (1979) Genetic Engineering: Principles and Methods, Vols. 1-4, Plenum Press, New York, which are expressly incorporated by reference herein.

References containing molecular biological techniques, procedures and protocols that are particularly useful in ciliated protozoa include J. Boothroyd et al. (eds.) Molecular Approaches to Parasitology, John Wiley & Sons, New York
5 and J. Gall (ed.) (1986) The Molecular Biology of Ciliated Protozoa, Academic Press, Inc., Orlando, FL., both of which are also expressly incorporated by reference herein.

Abbreviations and nomenclature, where employed, are deemed standard
10 in the field and commonly used in professional journals such as those cited herein.

Example I. Plasmid Construction: pBAB1 and pICH3

15 The pBTU1 plasmid containing a 3.7 kb Bgl II/Hind III fragment of the *Tetrahymena thermophila* β -tubulin gene, *BTU1*, was used to construct a derivative, pBAB1, in which the entire coding sequence was replaced with the coding sequence of the neomycin resistance gene, *neo1* (R. Kahn et al., Proc. Nat'l. Acad. Sci. USA 90:9295-9299 (1993)). Thus, the expression of *neo1* gene
20 on pBAB1 is driven by the flanking sequences of the *BTU1* gene. The plasmid pBAB1 was used to construct another derivative in which the *neo* coding region was replaced with the entire coding sequence of the *Ichthyophthirius* i-antigen (isolate G1) pre-protein. T. G. Clark et al., Proc. Natl. Acad. Sci. USA, 89: 6363-6367 (1992). To this end, the pBAB1 plasmid was amplified with the
25 NEO257 primer (5'-AGCCAGTCCCTTCCCGCTTCAGTGACAA-3' (SEQ ID NO:9) (provided by J. Bowen and M. Gorovsky, University of Rochester, NY) whose sequence encodes the antisense strand of the *neo1* gene, and primer BTN3 (5'-CGGGATCCAGCGAACTGAATCGGTCAGCT-3') (SEQ ID NO:10), corresponding to the 3' noncoding region of *BTU1* located immediately
30 downstream from the stop codon, TGA, and running in the same direction as the sense coding strand. The BTN3 primer contains a BamH I restriction site sequence at its 5' end, positioned immediately downstream from the stop codon,

TGA. The product of PCR amplification of pBAB1 using *NEO257* and *BTN3* primers contained the noncoding sequences of *BTU1*, an N-terminal half of the *neol* gene coding region and the vector sequence. A single *Hind* III site is located almost immediately downstream from the translation initiation codon, ATG, of the *neol* gene. The amplified product was digested with *Bam*H I and *Hind* III to obtain a smaller fragment containing the entire flanking sequences of *BTU1* and the plasmid vector. A plasmid carrying a 2 kb *Eco*R I genomic fragment of the *Ichthyophthirius* i-antigen gene was amplified with primers IC5 (5'CCCAGCTTGAAATATAATATTTTATTAATTTTAATT-3') (SEQ ID NO:11) and IC3 (5'-AGGGATCCTCACAATAAATAGAAAGAAATAA3') (SEQ ID NO:12). These primers amplified the entire coding sequence of the *Ich* i-antigen gene and introduced *Hind* III, and *Bam*H I restriction sites at positions encoding the N-terminal and C-terminal end of the protein respectively and corresponding to the positions of the same restriction sites present on the amplified fragment of pBAB1. This PCR product was digested with *Hind* III and *Bam*H I, and ligated to the *Bam*H I-*Hind* III digest of amplified pBAB1 to give pBICH3 plasmid, which contains the entire coding sequence of the i-antigen gene inserted in a correct translational frame between the regulatory sequences of the *BTU1* gene (see Figure 1). This new hybrid gene was designated *BTU1-4::LAG48[G1]*.

Example II. DNA-mediated Transformation of *T. thermophila*

Transformation

A *Tetrahymena thermophila* strain, CU522 (provided by Dr. Peter Bruns, Cornell University, Ithaca NY) was used as a transformation host. This strain carries a single substitution (K350M) in the β -tubulin, *BTU1* gene. This mutation was originally described for *Chlamydomonas reinhardtii* (Bolduc et al., Proc. Nat'l. Acad. Sci. USA 85:131-135 (1988)), and was later found to confer a similar phenotype in *T. thermophila*, namely, increased resistance to several microtubule depolymerizing drugs, such as oryzalin and colchicine, and

increased sensitivity to a microtubule stabilizing agent, paclitaxel (J. Gaertig et al., *Proc. Nat'l. Acad. Sci. USA* 91:4549-4553 (1994)).

Cells were grown in 50 ml of SPP medium (J. Gaertig, *Proc. Nat'l. Acad. Sci. USA*, 91:4549-4553 (1994)), supplemented with 100 U/ml penicillin, 100
5 $\mu\text{g/ml}$ streptomycin and 0.25 $\mu\text{g/ml}$ amphotericin B (SPPA medium), in 250 ml Erlenmayer flasks with shaking at 150 rpm at 30°C. Prior to transformation, the CU522 cells were grown to a density of about 6×10^5 cells/ml in SPPA with shaking at 30°C. Twenty-four hrs before transformation, 50 ml of growing cells were washed and suspended in 10 mM Tris-HCl (pH 7.5) buffer in the original
10 volume. After 4-5 hrs starving cells were counted again, cell concentration adjusted to 3×10^5 cells/ml and cells left at 30°C without shaking for another 18-20 hrs.

To target the *BTUI* gene derivatives to the endogenous *BTUI* locus of *T. thermophila*, either pBAB1 or pBICH3 plasmids were digested with Sac I and
15 Sal I restriction endonucleases to separate the insert (either *BTUI-2::neoI* or *BTUI-4::LAG48[G1]*) from the plasmid. Fifty micrograms of digested DNA was purified by a single phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by chloroform/isoamyl alcohol (24:1) extraction, precipitated with an equal volume of isopropyl alcohol in the presence of 0.15 M sodium acetate,
20 dried and resuspended at 1 mg/ml.

The transformation method used in this study targets genes into the somatic macronucleus of vegetative cells using biolistic bombardment (D. Cassidy-Hanley et al., *Genetics* 146:135-147 (1997)). Ten micrograms of linearized plasmid DNA was used to coat 60 mg of 0.6 μm gold particles (Bio-
25 Rad) using the Sanford Large Batch DNA Coating Method, as described by J.C. Sanford et al., *BioTechniques*, 3:3-16 (1991). An aliquot of 2.4×10^7 of starved cells was spun down at 600 x g for 3 min, washed with 45 ml of 10 mM Tris (pH 7.5) and resuspended in 3 ml of Tris buffer. One ml of cells was bombarded using 10 μg of DNA-coated gold particles at 900 psi using DuPont Biolistic
30 PDS-1000/He particle delivery system (Biorad). Bombarded cells were resuspended in 50 ml of SPPA, left for 2-3 hours at 30°C. Paclitaxel was added to the final concentration of 20 μM and cells were plated on microtiter plates

using 100 µl cells per well and plates were incubated in moist boxes at 30°C (in darkness, to prevent photobreakdown of paclitaxel). Wells containing paclitaxel-resistant transformants were apparent after 2-3 days of selection.

- To identify sequences integrated into the *BTUI* locus of *T. thermophila*,
 5 total genomic DNA was isolated from transformants using the fast urea method as described by J. Gaertig (Proc. Nat'l. Acad. Sci. USA, 91:4549-4553 (1994)) and used as a template for PCR with primers: *BTUI*-75 (5'-AAAAAATAAAAGTTTGAAAAAAA-3') (SEQ ID NO:13), sequence located 53-75 bp upstream to the ATG translation initiation codon in the *BTUI* 5' flanking region, and primer *BTUI*-3, (5'-GTTTAGCTGACCGATTTCAGTTCG-3') (SEQ ID NO:14), located close to the TGA stop codon in the 3' flanking region of *BTUI*. The resulting amplified products were digested with Nsi I restriction endonuclease and run on a TAE-agarose 0.7% gel.

15 ***Immunocytochemistry***

- For immunofluorescent detection of the *I. multifiliis* i-antigen in transgenic *Tetrahymena* cells, 10 mls of exponentially growing cultures (2×10^5 cells/ml) were harvested, washed with 10 mls of 10 mM Tris (pH 7.5) and resuspended in 0.5 ml. Cells were fixed with 3.5 mls of 2% paraformaldehyde in
 20 PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH. 6.9) for 30 minutes at room temperature. Fixed cells were washed once with 3 mls of PHEM buffer and 2 times with 3 mls of modified phosphate buffered saline, PBS (130 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 10 mM EGTA, 2 mM $MgCl_2$, pH. 7.2) and resuspended in 0.5 ml. For antibody
 25 labeling, 100 µl of fixed cells were washed 3 times for 10 minutes with 3 mls of PBS-BT, (3% bovine serum albumin, 0.1% Tween 20 in PBS), and concentrated in 100 µl. One µl of affinity-purified rabbit antiserum directed against *Ichthyophthirius* G1 i-antigen (T.L. Lin et al., J. Protozool., 39:457-463 (1992)), was added, followed by incubation overnight at 4°C, three washes in PBS-BT and
 30 incubation with the detection antibodies (at 1:100 dilution) for 45 minutes at room temperature.

Secondary antibodies were goat anti-rabbit IgG coupled to FITC (Zymed). Labeled cells were washed 3 times with PBS, concentrated in 0.1 ml of PBS plus 10 µl of DABCO mounting medium (100 mg/ml 1,4-diazobicyclo-[2,2,2]-octane, Sigma Chemical Co., dissolved in 90% glycerol in PBS). To mount, 5 µl of cells
5 were combined with 5 µl of DABCO medium, covered with a cover-slip and sealed with nail polish. Slides were examined with a Bio-Rad MRC 600 Laser Scanning Confocal Microscope at the UGA Center for Advanced Ultrastructural Research. Sets of optical sections of individual cells were processed to obtain complete 3-D reconstructions.

10

Immunoblotting

Protein extracts were prepared from $1-2 \times 10^6$ cells taken from exponentially growing cultures. Cells were spun down at 2000 rpm for 5 minutes, washed with 10 mls of ice-cold 10 mM Tris, pH 7.5, and resuspended in 125 µl
15 of ice-cold Tris buffer supplemented with a mixture of protease inhibitors which included 0.5 µg/ml leupeptin, 10 µg/ml E-64, 10 µg/ml chymostatin and 12.5 µg/ml antipain (A. Turkewitz, personal communication, all inhibitors from Sigma), combined with 125 µl of boiling 2xSDS-PAGE sample buffer and boiled for 5-10 min. Ten µl of extracts were loaded on a 10% SDS-PAGE minigel and
20 proteins transferred on nitrocellulose using semi-dry transfer system (Biorad). The filter was blocked for 2 hr in PBS-T buffer containing 5% dried milk, followed by incubation with the anti-*I. multifiliis* i-antigen antibodies (1:10000) overnight at 4°C. The filter was washed extensively with PBS with 0.1% Tween-20 (PBST) and incubated in the same buffer containing the goat-anti-rabbit IgG
25 antibodies conjugated to alkaline phosphatase (Bio-Rad) for 1 hr at room temperature. The membrane was washed in PBST and developed using NBT/BCIP (Bio-Rad) as described by M. Gorovsky, *J. Protozool.*, 20:19-25 (1973)

Immobilization Assay

Growing *Tetrahymena* cells were washed with 10 mM Tris, pH 7.5 and resuspended at 2000 cells/ml. Cells were incubated with a series of dilutions of monospecific rabbit polyclonal antiserum against the parasite i-antigen 48 kD protein on microtiter plates at 1000 cells/ml in 100 microliter/well aliquots. After 15-60 minutes wells were scored under a dissecting scope. The behaviors of both *BTU1-4::LAG48[G1]* transformants and controls (*neol* transformants) were examined, and the percentage of immobilized cells was estimated.

10 *Results*

Cells were selected for resistance to either paclitaxel (30 μ M) or paromomycin (120 μ g/ml). As shown in Table I, transformants were readily obtained following selection with either drug, but were not detected in a mock transformation experiment. Furthermore, random clones that had been selected for growth in paclitaxel were all found to be cross-resistant to paromomycin (n=40), and nearly all transformants originally selected with paromomycin, produced clones resistant to 30 μ M paclitaxel (89%, n=71). Thus, acquisition of the transformed phenotype (in most, if not all cases) resulted from disruption of the host *btu1-1K350M* gene, and negative paclitaxel selection based on *BTU1* gene loss-of-function was nearly as effective as a positive selection based on paromomycin resistance conferred by the transgene.

Table I. Transformation of *T. thermophila* using biolistic bombardment

	Targeting Fragment	Selection method	Frequency (transf./ μ g DNA)
5	<i>BTU1-2::neol</i>	tx*	115
	<i>BTU1-2::neol</i>	pm [†]	204
	No DNA	tx	0
10	No DNA	pm	0
	<i>BTU1-2::neol</i>	tx	37
	<i>BTU1-4::LAG[G1]</i>	tx	31
	No DNA	tx	0

15 *30 μ M paclitaxel. [†]120 μ g/ml paromomycin.

Unlike the bacterial *neo* gene, the product of the i-antigen gene of *I. multifiliis* is not inherently selectable. Nonetheless, as shown in Table I, paclitaxel-resistant transformants were obtained with a frequency comparable to that seen with the control *BTU1-2::neol* gene construct. Western blotting studies (Fig. 5) using polyclonal antiserum against affinity purified i-antigens of *Ichthyophthirius* showed that a protein of apparent MW of 50 kDa was detected in all transformants carrying the *LAG48[G1]* gene (lanes 1-5), but not in a control cell line transformed with *neol* (lane 6). The size of the detected protein was in close agreement with the MW of the corresponding surface antigen of *Ichthyophthirius*. An additional band of about 100 kDa seen in both *BTU1-4::LAG48[G1]* and control extracts, most likely represents an endogenous *Tetrahymena* protein that shares immunological determinants with the parasite antigen.

It should be noted that because the macronucleus of *T. thermophila* is polyploid and contains about 45 copies of each chromosome, only partial replacement of endogenous genes occurs following initial transformation. Nevertheless, chromosomes are distributed to daughter nuclei through an imprecise form of allelic segregation known as amitosis, and complete replacement of endogenous genes by transgenic copies can be achieved by continuous growth in selective media. In this case, PCR analysis revealed that about one half of

endogenous copies of the *BTUI* gene were replaced by the *LAG48[G1]* transgene during early stages of selection. Following growth in the presence of increasing concentrations of paclitaxel, however, endogenous copies of the *BTUI* gene could no longer be detected, and a corresponding increase in the copy number of *LAG48[G1]* was seen, indicating that they had lost all copies of the endogenous *BTUI* allele.

In *Ichthyophthirius*, the 48-kDa protein is a major component of the cell surface and is bound to ciliary and plasma membranes through a glycosylphosphatidylinositol (GPI) anchor. Nevertheless, the sequence elements responsible for membrane targeting in ciliates are not well-understood, and while *Ichthyophthirius* and *Tetrahymena* are taxonomically related, it was not known whether signal peptides from such widely diverged species could function interchangeably.

Transformed cell lines were therefore fixed (without permeabilization), reacted with antibodies against the 48-kDa antigen and analyzed for the presence of the *I. multifiliis* protein on their surface by indirect immunofluorescence using confocal microscopy. Strong labeling of oral and somatic cilia was observed (Fig. 6A, 6B and 6C), wherein somatic cilia are shown in Fig. 6A and oral cilia are shown in Fig. 6C. Surface cortical fluorescence was seen in the form of longitudinal rows (shown in Fig. 6B). Dividing cells showed labeling in both the preexisting and newly formed oral apparatus, indicating that as the new structure is formed, parasite antigens are rapidly inserted into the cell membrane (Figure 6C). Control cells (transformed with *neoI*) showed no obvious labeling (Figure 6D). Fluorescence was only seen in controls when cells were permeabilized with methanol prior to fixation with paraformaldehyde. This appeared as a generalized background staining, and was not surprising given the presence of a cross-reacting (100 kDa) band in Western blots of control cell extracts.

A further demonstration of the presence of *I. multifiliis* proteins on the surface of *Tetrahymena* came from immobilization assays with living cells. In the immobilization assay, at antisera dilutions of 1:100 or less, rabbit antisera against the 48-kDa protein caused an immediate reduction in swimming velocity of the *BTUI-4::LAG48[G1]* transformants, followed by a complete loss of motility within 50 minutes. In contrast, *neoI* transformants showed no immediate effect, but eventually

became immobilized after 90 minutes. The difference in the response of *BTUI-4::LAG48[G1]* versus *neoI* transformants indicates that *I. multifiliis* antigens are accessible to antibodies at the surface of live cells. Furthermore, because immobilizing antibodies recognize 3-dimensional epitopes on the 48 kDa protein, the antigen itself is likely to assume a native conformation in *Tetrahymena*.

Example III. Expression of Green Fluorescent Protein in *Tetrahymena*

Using the *Tetrahymena BTUI* targeting system (Examples I and II), a modified version of the G1 i-antigen was constructed in which the green fluorescent protein (GFP) tag coding sequence was inserted in a correct frame into the G1 i-antigen of *I. multifiliis*, about 10 amino acids downstream from the site of cleavage of the N-terminal signal peptide. The G1-GFP construct was thereby targeted to the *BTUI* locus, and transformants were selected with paclitaxel. The fusion protein was detected inside transformed cells using Western blotting and anti-gfp antibodies. A fraction of the fusion protein was found to be associated with the Triton X-114 extractable membranes of transformant cells, although no fusion protein was found on the cell surface.

Example IV. Expression of Malaria Surface Protein in *Tetrahymena*

Malaria is caused by parasitic protozoa of the genus *Plasmodium*, and is responsible for 2 million deaths a year worldwide. Current control efforts are focused on the mosquito vector, and treatment of the disease with anti-malarial drugs. No vaccine is yet available. Over the past four decades the parasite has become resistant to many anti-malarial drugs, and an effective vaccine is seen as best hope for controlling morbidity and mortality caused by this pathogen.

Most of the candidate molecules from *Plasmodium* suggested for inclusion in a vaccine are proteins that bind to receptors on host target cells. Necessary for vaccine development is the identification and characterization of the role played by these proteins. Unfortunately, this effort has been hampered by difficulties in procuring enough parasite protein to allow thorough study. Several heterologous

expression systems have been utilized, but none has proven to be ideal, especially for the production of functional protein.

In this preliminary study, the *Tetrahymena BTU1* targeting system (Examples I and II) was evaluated for use in expression of the *Plasmodium falciparum* surface proteins implicated in interactions with the host cells. A 1,900 bp region of a putative *P. falciparum* erythrocyte binding protein, EBL-1, was directly inserted in frame, at a *NsiI* restriction site, into the coding sequence of the G1 i-antigen of *I. multifiliis* flanked by the *BTU1* targeting sequences. The resulting construct had a coding region encoding a chimeric fusion protein having the N-terminal portion of the i-antigen (representing about 2/3 of the i-antigen protein), followed by the EBL-1 domain encoded by the 1,900 bp region, followed by the C-terminal portion of the i-antigen (representing about 1/3 of the i-antigen protein), under control of the *Tetrahymena BTU1* promoter.

This construct was expressed in *Tetrahymena*, demonstrating that the presence within the *P. falciparum* coding sequence of codons that are very rare in *Tetrahymena* did not abrogate expression. However, this EBL1::G1 fusion protein was expressed in *Tetrahymena* as a cytosolic protein, and no association with the cell membrane or the cell surface was observed despite the fact that the N-terminal and C-terminal targeting sequences of G1 i-antigen were present in the fusion protein. This result could be explained by improper folding of the multi-domain chimeric fusion protein; insertion of the EBL-1 coding sequence into the middle of the G1 i-antigen coding sequence arbitrarily divided the i-antigen sequence into two portions, possibly disrupting folding of the protein or otherwise interfering with the production of a protein that was competent to translocate into or across a membrane. A second experiment replacing the i-antigen coding sequence (between the two targeting sequences) with the EBL-1 domain instead of simply inserting it within the i-antigen coding sequence will provide more insight into targeted expression of EBL-1 in *Tetrahymena*.

Example V. Deletion of GPI Anchor Modification Domain

A truncated version of the G1 i-antigen gene from *I. multifiliis* was prepared
5 in which the C-terminal domain implicated in the GPI anchor addition was deleted.
The truncated gene was expressed in *Tetrahymena* as in Example II. Figure 7 shows
Western blots of total protein extracts of transformant cells (Fig. 7(a)) and total
culture medium from which transformant cells were removed (Fig. 7(b)). The first
four lanes from the left contained either cells (Fig. 7(a)) or culture medium (Fig.
10 7(b)) of four independent transformants of *T. thermophila* in which a truncated
version of the G1 antigen lacking the GPI-anchoring C-terminal domain was
incorporated into the *BTUI* locus. Controls contained either the *neo* gene or a full-
length G1 i-antigen of *I. multifiliis* expressed in the *BTUI* locus. The amount of
culture medium loaded (Fig. 7(b)) corresponds to about 1/30 of the amount of cells
15 loaded (Fig. 7(a)). The truncated protein was expressed at essentially the same level
observed for surface-targeted expression of the gene (Example II) (Figure 7, top).
However, about 1/2 of total expressed truncated G1 i-antigen protein was found in the
culture medium, in contrast to the full length G1 i- antigen protein which was not
detected in the culture medium. Thus, the elimination of the GPI anchor domain
20 caused the i-antigen to be secreted out of the cell, as expected.

Example VI. Expression of Chicken Ovalbumin in *Tetrahymena*

To test the feasibility of *Tetrahymena thermophila* for expression of
25 vertebrate proteins, we prepared two constructs in which the region of the coding
sequence of chicken ovalbumin protein (amino acids 139-385; GenBank Acc.
Numbers M34346 and M25173; J. Catterall et al., *Nature* 275:510-513 (1978)) was
fused to an N-terminal signal peptide of either the gp-72 surface protein of *T. cruzi*
(Tryp./OVA) or of the G1 antigen of *I. multifiliis* (Ich/OVA) subcloned between the
30 flanking sequences of the *BTUI* gene of *T. thermophila*. These constructs were
integrated into the *BTUI* locus using biolistic transformation of the paclitaxel-
sensitive host cells, and protein extracts of several paclitaxel-resistant transformants

were analyzed by a Western blot using anti-chicken ovalbumin antibodies (Sigma Chemical Co.). Two protein bands close to the expected molecular weight of about 30 kD were detected in transformants but not in the negative control cells in which the *BTUI* locus was transformed using the bacterial neomycin resistance gene (*neo*) (Fig. 8). The presence of two bands may be the result of posttranslational modifications or proteolysis in *Tetrahymena*. Interestingly, the fusion protein containing the signal peptide of *T. cruzi* gp72 protein was expressed at several fold higher level compared to the fusion protein with the *I. multifiliis* signal peptide. Also, it is striking that the levels of expression are extremely reproducible among individual transformants.

**Example VII. Immune Response of Channel Catfish to Live Vaccine:
Transformed *Tetrahymena* Expressing Full-length or Truncated *I. multifiliis*
(Serotype A) 48 kD I-antigen Protein and Heterologous Challenge with
I. multifiliis (Serotype D)**

The *LAG48[G1]* gene of *Ichthyophthirius multifiliis* G1 encodes the GPI anchored 48- kDa i-antigen. The extreme 3' region of the gene encodes a stretch of 14 mostly hydrophobic amino acids separated by a short spacer from three small amino acids (CAS). This sequence encodes the protein's GPI anchor addition site. *Tetrahymena thermophila* cells transformed with the entire *LAG48[G1]* gene produce an intact i-antigen anchored to the cells' surface. *Tetrahymena* cells transformed with a modified *LAG48[G1]* gene construct lacking the 3' sequence which includes the GPI addition site would be expected to produce a truncated protein lacking the GPI anchor.

Tetrahymena cells were transformed with either the entire *Ichthyophthirius* G1 48- kDa i-antigen protein, or a truncated gene sequence which encodes the i-antigen protein lacking 19 amino acids at the carboxy terminus. Transformants encoding the intact or C-terminal truncated i-antigen were grown in standard *Tetrahymena* growth medium. Cell pellets and supernatant fluids were collected at the time points indicated. I-antigen was detected in cell cytosol, cell membrane or cell culture supernatants by Western blots using rabbit antisera against affinity

purified *Ichthyophthirius* G5 i-antigen (see Fig. 9). It is clearly seen that the truncated protein is secreted into the culture medium.

Groups of channel catfish (6 fish per group) were immunized by bath exposure (10^6 or 10^5 cells/fish) or intraperitoneal injection with *T. thermophila* transformants (10^6 , 10^5 , or 10^4 cells/fish) producing intact or truncated i-antigen. A third group of fish was immunized with membrane protein extracts (1 mg or 0.1 mg/fish) from *T. thermophila* producing the full length protein.

Immunization by bath exposure to Tetrahymena transformants. Two groups of fish (6 fish in each group) were immunized by bath exposure. The fish were exposed to either 10^6 or 10^5 cells /fish for a period of 24 hours. Two immunogens were used: 1) transformed *Tetrahymena* cells expressing the entire *Ichthyophthirius* G1 48-kDa protein, and 2) transformed cells secreting a truncated form of the i-antigen lacking the GPI anchor. Fish in the control group were exposed to *Tetrahymena* transformants expressing the *neol* gene product. Fish were exposed twice at a 30 day interval and challenged 30-60 days after the last immunization with the G5 *Ichthyophthirius* isolate. There were no significant differences (z test) between test and control groups (see Table 1). Immunized fish were challenged with a heterologous strain of *Ichthyophthirius* expressing a different i-antigen (serotype D; G5 isolate) than that produced by the recombinant *Tetrahymena* used for vaccination. It is expected that challenge with a strain of *Ichthyophthirius* producing an i- antigen homologous to the G1 48 i-antigen would show increased levels of protection.

TABLE 1. Vaccination by bath exposure

	Immunogen	Dose (cells/fish)	Number of fish challenged	Number of fish surviving	% survival	RSP ³	MDD ⁴ ± SD ⁵
5	Neo control	10 ⁶	5	2	40	N.A.	17.0 ± 1.0
	TG1 ¹	10 ⁵	5	3	60	33.3	18.5 ± 2.1
	TG1	10 ⁶	6	3	50	25.0	13.3 ± 4.9
	sTG1 ²	10 ⁵	6	3	50	25.0	21.0 ± 4.6
10	sTG1	10 ⁶	6	4	66.7	40.0	17.0 ± 1.4

¹ *Tetrahymena* expressing intact membrane form of *Ichthyophthirius* G1 i-antigen.

² *Tetrahymena* secreting truncated form of G1 i-antigen.

³ Relative Survival Percent = 1 - (number of dead fish in test group/number of dead fish in control group) x 100%

15 ⁴ Mean days to death

⁵ Standard deviation

Immunization by injection of Tetrahymena transformants. Fish in each group were injected intraperitoneally with 10⁶, 10⁵, or 10⁴ live transformed *Tetrahymena* cells/fish. The same immunogens and controls were tested as in the immersion vaccinations. Fish were injected two times at a 30 day interval, and challenged 21 days after the last immunization with G5 *Ichthyophthirius*. A greater degree of protection was elicited in immunized fish compared to controls (Table 2).

TABLE 2. Vaccination by injection

	Immunogen	Dose (cells/fish)	Number of fish challenged	Number of fish surviving	% survival	RSP ³	MDD ⁴ ± SD ⁵
5	Neo control	10 ⁵	6	2	33.3	N.A.	15.3 ± 3.6
	TG1 ¹	10 ⁶	5	3	60	44.5	19.0 ± 2.8
	TG1	10 ⁵	5	4	80	58.4	15.0 ± 0.0
	TG1	10 ⁴	6	2	33.3	0	14.0 ± 1.4
	sTG1 ²	10 ⁶	6	5	83.3	50.0	21.0 ± 4.6
10	sTG1	10 ⁵	6	3	50.0	25.0	20.0 ± 5.7

¹ *Tetrahymena* expressing intact membrane form of *Ichthyophthirius* G1 i-antigen.

² *Tetrahymena* secreting truncated form of G1 i-antigen.

³ Relative Survival Percent = 1 - (number of dead fish in test group/number of dead
15 fish in control group) x 100%

⁴ Mean days to death

⁵ Standard deviation

Serum antibody production. Fish serum antibody responses against
20 recombinant G1 *Ichthyophthirius* i-antigen were determined by ELISA at 2, 4, and 6
weeks after immunization. Serum antibodies from immunized fish were detected
with a sandwich ELISA technique that used wells coated with a cross-reactive rabbit
antibody against *Ichthyophthirius* G5 i-antigen to capture recombinant G1 i- antigen
produced in transformed *Tetrahymena*. Sera from test and control fish were added to
25 wells and antibodies that bound to the captured i-antigen were detected using an
alkaline phosphatase labeled mouse mAb against the immunoglobulin heavy chain of
channel catfish. ELISA controls consisted of antibody-coated wells reacted with
membrane protein from *Tetrahymena* cells transformed with the *neol* gene.

Fish injected with *Tetrahymena* membrane protein produced high levels of serum antibody against the recombinant i-antigen. The antibody response elicited by fish immunized with live cells was almost an order of magnitude lower. The antibody response of fish immunized by bath or i.p. injection with live cells secreting recombinant i-antigen was approximately two-fold greater than the antibody response of fish immunized with *Tetrahymena* producing the membrane-bound, intact i-antigen. In Fig. 10, the differences in antibody production between fish immunized with the (a) membrane associated or (b) secreted form of the i-antigen are shown. These results suggest that live cells secreting antigen are more efficacious in eliciting the production of serum antibodies. The mucosal antibody response was not determined in these experiments.

Example VIII.

Immune Response of Channel Catfish to Live Vaccine:

Transformed *Tetrahymena* Expressing Full-length or Truncated *I. multifiliis* (Serotype A) 48 kD I-antigen Protein and Homologous Challenge with *I. multifiliis* (Serotype A)

Tetrahymena cells were transformed with either the entire *Ichthyophthirius* G1 48- kDa i-antigen protein, or a truncated gene sequence which encodes the i-antigen protein lacking 19 amino acids at the carboxy terminus as in Example VII.

Groups of channel catfish (70 fish per group) were vaccinated by intraperitoneal injection with 10^6 *T. thermophila* transformants producing intact or truncated i-antigen. A third group of fish (control group) was vaccinated with *T. thermophila* transformants expressing *neo*. No adjuvant was used in any of the vaccinations. The fish were boosted 2 weeks following the initial injection and bled at 3 weeks following the initial injection. Sera from 3 fish per group were pooled.

A 96 well ELISA plate was seeded with a homologous strain (i.e., serotype A) of *I. multifiliis* (strain NY1, a G1 isolate), 200 cells per well. Fish sera were serially diluted and added to the wells, and the effect on the motility of *I. multifiliis* was observed. Immobilization of *I. multifiliis* was immediately evident at serum dilutions of 1:20, and at higher concentrations the organisms exhibited clumping (see

Fig. 11). Sera from the control group did not cause any detectable change in motility of *I. multifiliis*.

As another control, additional wells were seeded with a heterologous strain of *I. multifiliis* (a G5 isolate). The motility of these organisms was not affected by sera
5 from any of the groups of vaccinated fish, confirming that the immobilization epitopes on *I. multifiliis* i-antigens are highly specific.

For comparison, two other groups of fish were vaccinated with purified subunit proteins produced from recombinant *Tetrahymena* (either the full-length 48 kD i-antigen protein or the C-terminal truncated version). The subunit proteins were
10 adjuvanted with Freund's Complete Adjuvant. In a plate assay similar to the one described above using the homologous strain of *I. multifiliis*, some immobilization was observed but not to the degree caused by the "live vaccine." This observation lends support to the expectation that the "live vaccine" will prove to be more efficacious than the analogous protein subunit vaccine.

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The complete disclosures of all patents, patent applications including provisional patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and
20 examples have been provided for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described; many variations will be apparent to one skilled in the art and are intended to be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. A recombinant protein expression system comprising a transgenic protozoan host cell that is resistant to paclitaxel, the host cell comprising a heterologous nucleic acid encoding a polypeptide, wherein the recombinant protozoan host cell is selectable by negative selection using paclitaxel.
2. The recombinant protein expression system of claim 1 wherein the heterologous nucleic acid is derived from an organism that has an AT-rich genome.
3. The recombinant protein expression system of claim 1 wherein the protozoan host cell is a *Tetrahymena* host cell comprising, prior to genomic integration of the heterologous nucleic acid, a *btu1-1K350M* β -tubulin allele.
4. A recombinant protein expression system comprising a transgenic ciliated protozoan host cell comprising a heterologous protein displayed on the plasma membrane surface of the host cell.
5. The recombinant protein expression system of claim 4 wherein the transgenic ciliated protozoan host cell is a *Tetrahymena* host cell.
6. The recombinant protein expression system of claim 4 wherein the surface-displayed heterologous protein comprises a GPI anchor encoded by a portion of an i-antigen-encoding nucleotide sequence from *I. multifiliis*.
7. An expression vector comprising a 5' flanking region followed by a heterologous nucleic acid encoding a polypeptide comprising at least one targeting amino acid sequence encoded by a portion of an i-antigen-encoding nucleotide sequence from *I. multifiliis*, followed by a 3' flanking region, wherein at least a portion of each of the 5' flanking region and the 3' flanking region is complementary to an endogenous gene of an intended host, so as to allow for integration into endogenous gene by way of homologous recombination.

8. The expression vector of claim 7 wherein the 5' flanking region further comprises a promoter region operably linked to the heterologous nucleic acid sequence.
9. The expression vector of claim 7 wherein the 5' flanking region and the 3' flanking region each comprises a nucleic acid sequence selected from at least a portion of the *Tetrahymena* genes *HHF1*, *rpl29*, *BTU1*, *BTU2*, *SerH3* and the gene encoding actin.
10. The expression vector of claim 7 wherein the at least one targeting amino acid sequence comprises a GPI cleavage/attachment sequence derived from an i-antigen protein of *I. multifiliis*.
11. A transgenic *T. thermophila* comprising at least a portion of an *I. multifiliis* i-antigen protein.
12. The transgenic *T. thermophila* of claim 11 wherein the portion of the *I. multifiliis* i-antigen protein comprises a targeting amino acid sequence.
13. The transgenic *T. thermophila* of claim 12 wherein the targeting amino acid sequence comprises at least one of an N-terminal targeting sequence and a GPI cleavage/attachment sequence.
14. The transgenic *T. thermophila* of claim 11 comprising an *I. multifiliis* antigen protein displayed on the surface of its plasma membrane.
15. A transgenic cell comprising a heterologous protein comprising at least one targeting amino acid sequence encoded by an i-antigen-encoding nucleotide sequence from *I. multifiliis*.
16. The transgenic cell of claim 15 wherein the targeting amino acid sequence comprises at least one of an N-terminal targeting sequence and a GPI cleavage/attachment sequence

17. The transgenic cell of claim 15 wherein the heterologous protein is displayed on the surface of the plasma membrane of the cell.

18. A method for making a polyclonal antibody comprising:

expressing an antigenic polypeptide on the surface of the plasma membrane of a transgenic protozoan host cell;

administering the transgenic protozoan host cell to an animal to generate an antibody response to the antigenic polypeptide; and

isolating the antibody from the animal.

19. A method for making a polyclonal antibody comprising:

expressing an antigenic polypeptide on the surface of the plasma membrane of a transgenic protozoan host cell;

cleaving the antigenic polypeptide from the surface of the host cell;

isolating the antigenic polypeptide;

administering the antigenic polypeptide to an animal to generate an antibody response to the antigenic polypeptide; and

isolating the antibody from the animal.

20. A method for detecting antibodies to an antigenic polypeptide comprising:

expressing the antigenic polypeptide on the surface of a transgenic protozoan host cell;

exposing the protozoan host cell to an antibody; and

determining whether the swimming behavior of the protozoan host cell is altered;

wherein alteration of the swimming behavior of the protozoan host cell is indicative of the presence of antibodies to the antigenic polypeptide.

21. The method of claim 20 wherein the host cell is immobilized.

22. The method of claim 20 wherein the antigenic polypeptide is a pathogenic parasite polypeptide, and wherein the host cell is exposed to the bodily fluid of a patient suspected of being infected with the parasite.
23. A method for screening drugs for the ability to bind a polypeptide comprising:
expressing the polypeptide on the surface of a transgenic protozoan host cell;
exposing the host cell to a drug; and
determining whether the swimming behavior of host cell is altered;
wherein an alteration in the swimming behavior of the host cell is indicative of binding of the drug to the polypeptide.
24. The method of claim 23 wherein the host cell is immobilized after exposure to the drug.
25. The method of claim 23 wherein the polypeptide is a human polypeptide.
26. A vaccine comprising a transgenic nonpathogenic immunogenic protozoan comprising a surface-displayed antigenic polypeptide.
27. The vaccine of claim 26 comprising a live vaccine.
28. The vaccine of claim 26 comprising a killed vaccine.
29. The vaccine of claim 26 wherein the transgenic nonpathogenic immunogenic protozoan is a transgenic nonpathogenic immunogenic ciliated protozoan
30. The vaccine of claim 29 wherein the transgenic nonpathogenic immunogenic ciliated protozoan is *Tetrahymena*.
31. The vaccine of claim 30 wherein the *Tetrahymena* comprises a *btu1-1K350M* locus into which a heterologous nucleic acid encoding the antigenic polypeptide has been inserted.

32. The vaccine of claim 26 wherein the antigenic polypeptide comprises at least an antigenic portion of an *I. multifiliis* i-antigen protein.
33. A method for stimulating an immune response in a vertebrate comprising delivering to the vertebrate a transgenic nonpathogenic immunogenic protozoan comprising a surface-displayed antigenic polypeptide.
34. The method of claim 33 wherein the transgenic nonpathogenic immunogenic protozoan is a *Tetrahymena*.
35. The method of claim 33 wherein the antigenic polypeptide comprises at least an antigenic portion of an *I. multifiliis* i-antigen protein.
36. A recombinant method for producing a polypeptide comprising:
- (a) providing a protozoan host cell that is resistant to paclitaxel;
 - (b) introducing a heterologous nucleic acid encoding the polypeptide into the protozoan host cell to yield a transgenic protozoan host cell that is selectable by negative selection using paclitaxel; and
 - (c) expressing the polypeptide in the transgenic protozoan host cell.
37. The recombinant method of claim 36 wherein the protozoan host cell provided in step (a) is *Tetrahymena*.
38. The recombinant method of claim 37 wherein the *Tetrahymena* comprises a *btu1-1K350M* β -tubulin allele.
39. The recombinant method of claim 36 wherein the polypeptide is displayed on the plasma membrane of the transgenic protozoan host cell.
40. The recombinant method of claim 39 further comprising:
- (d) cleaving the polypeptide from the plasma membrane of the transgenic protozoan host cell.

41. The recombinant method of claim 36 wherein the polypeptide comprises at least one targeting amino acid sequence encoded by an i-antigen-encoding nucleotide sequence from *I. multifiliis*.
42. The recombinant method of claim 36 wherein the polypeptide is an antigenic polypeptide.
43. The recombinant method of claim 36 further comprising:
(d) isolating the polypeptide from the transgenic protozoan host cell.
44. A recombinant method for producing a polypeptide comprising:
(a) providing a ciliated protozoan host cell;
(b) introducing a heterologous nucleic acid encoding the polypeptide into the protozoan host cell to yield a transgenic ciliated protozoan host cell; and
(c) expressing the polypeptide in the transgenic ciliated protozoan host cell such that it is displayed on the plasma membrane surface of the host cell.
45. The recombinant method of claim 44 wherein the polypeptide comprises at least one targeting amino acid sequence encoded by an i-antigen-encoding nucleotide sequence from *I. multifiliis*.
46. The recombinant method of claim 44 further comprising:
(d) cleaving the polypeptide from the plasma membrane surface of the transgenic host cell.
47. The recombinant method of claim 44 wherein the polypeptide is an antigenic polypeptide.
48. The recombinant method of claim 44 wherein the transgenic ciliated protozoan host cell is *Tetrahymena*.

49. A recombinant method for producing a polypeptide comprising:
- (a) providing a host cell;
 - (b) introducing a heterologous nucleic acid encoding the polypeptide into the host cell to yield a transgenic host cell, wherein the polypeptide comprises at least one targeting amino acid sequence encoded by an i-antigen-encoding nucleotide sequence from *I. multifiliis*.; and
 - (c) expressing the polypeptide in the transgenic host cell.
50. The recombinant method of claim 49 wherein the at least one targeting amino acid sequence comprises at least one of an N-terminal targeting sequence and a GPI cleavage/attachment sequence.
51. The recombinant method of claim 49 wherein the at least one targeting amino acid sequence is derived from a 48 kD i-antigen of *I. multifiliis*.
52. The recombinant method of claim 49 wherein the at least one targeting amino acid sequence is derived from a 55 kD i-antigen of *I. multifiliis*.

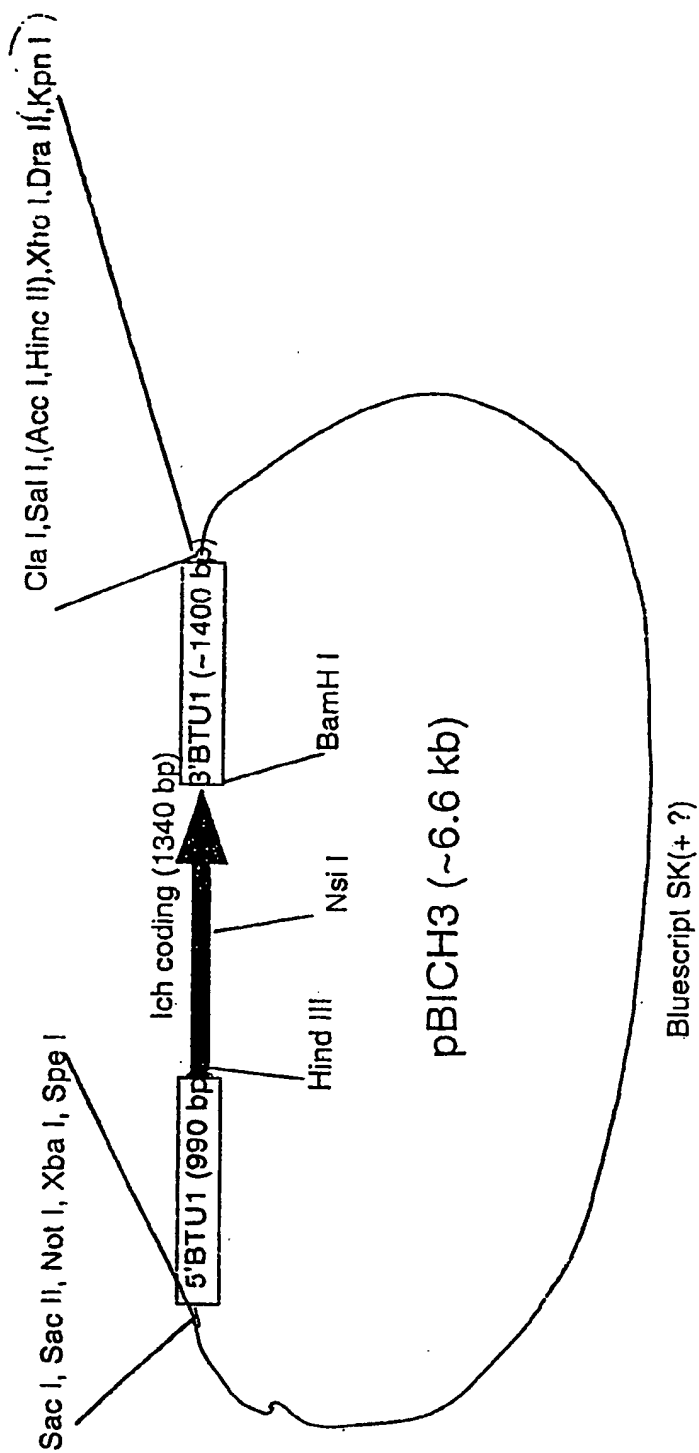


Figure 1

-> Restriction Map of BTU1::ukol

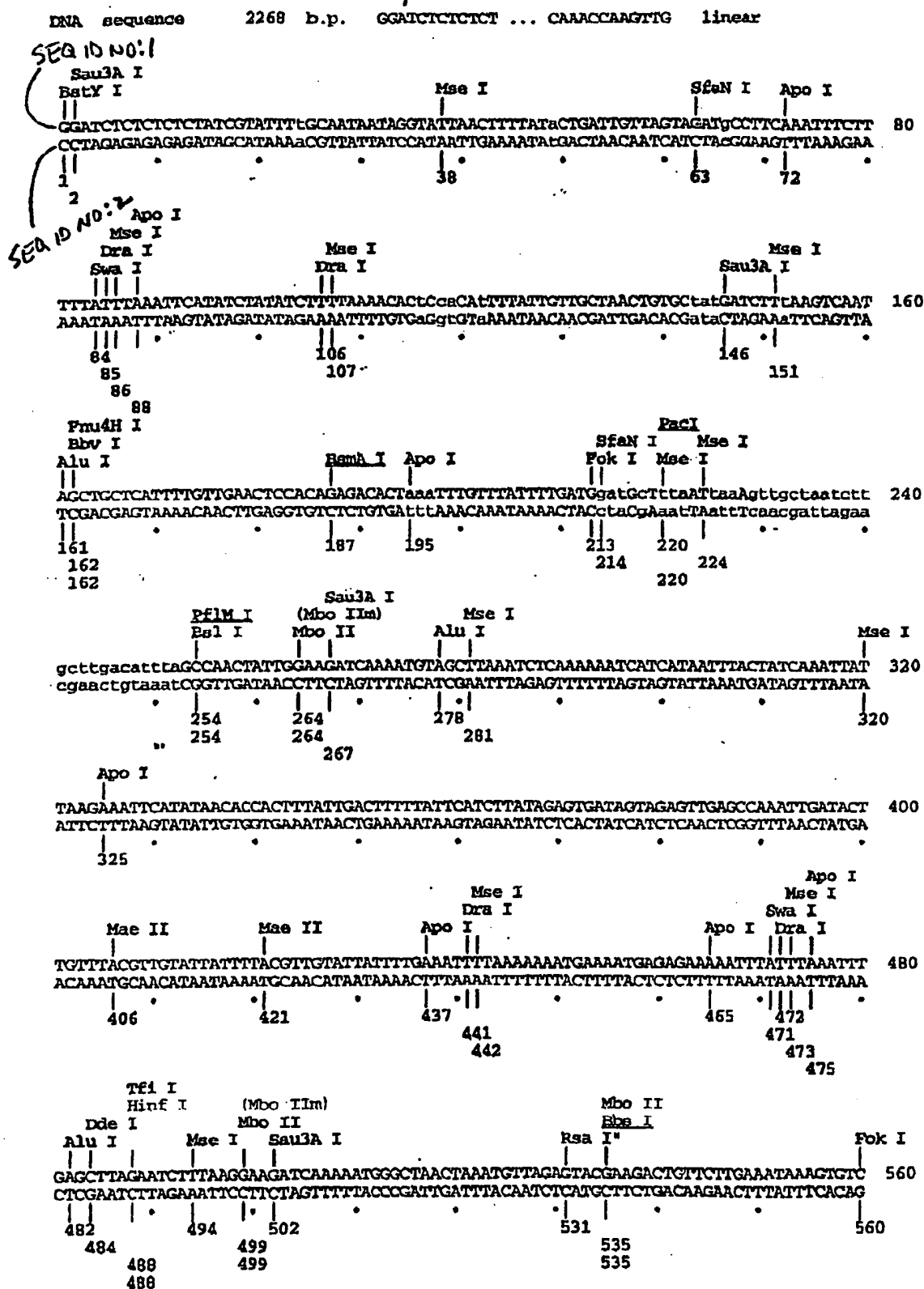


Fig 2(a)

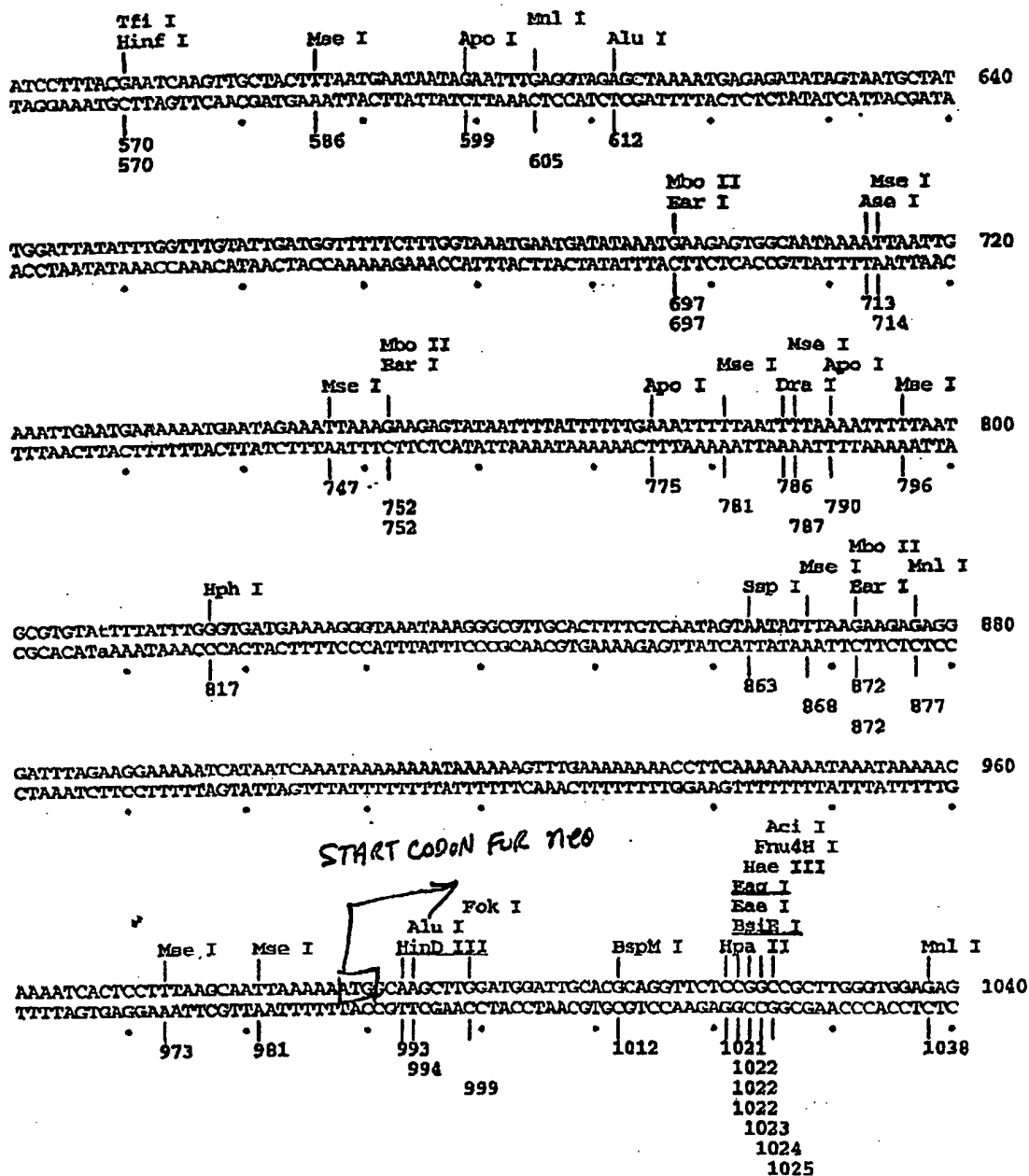


Fig. 2(a) cont'd.

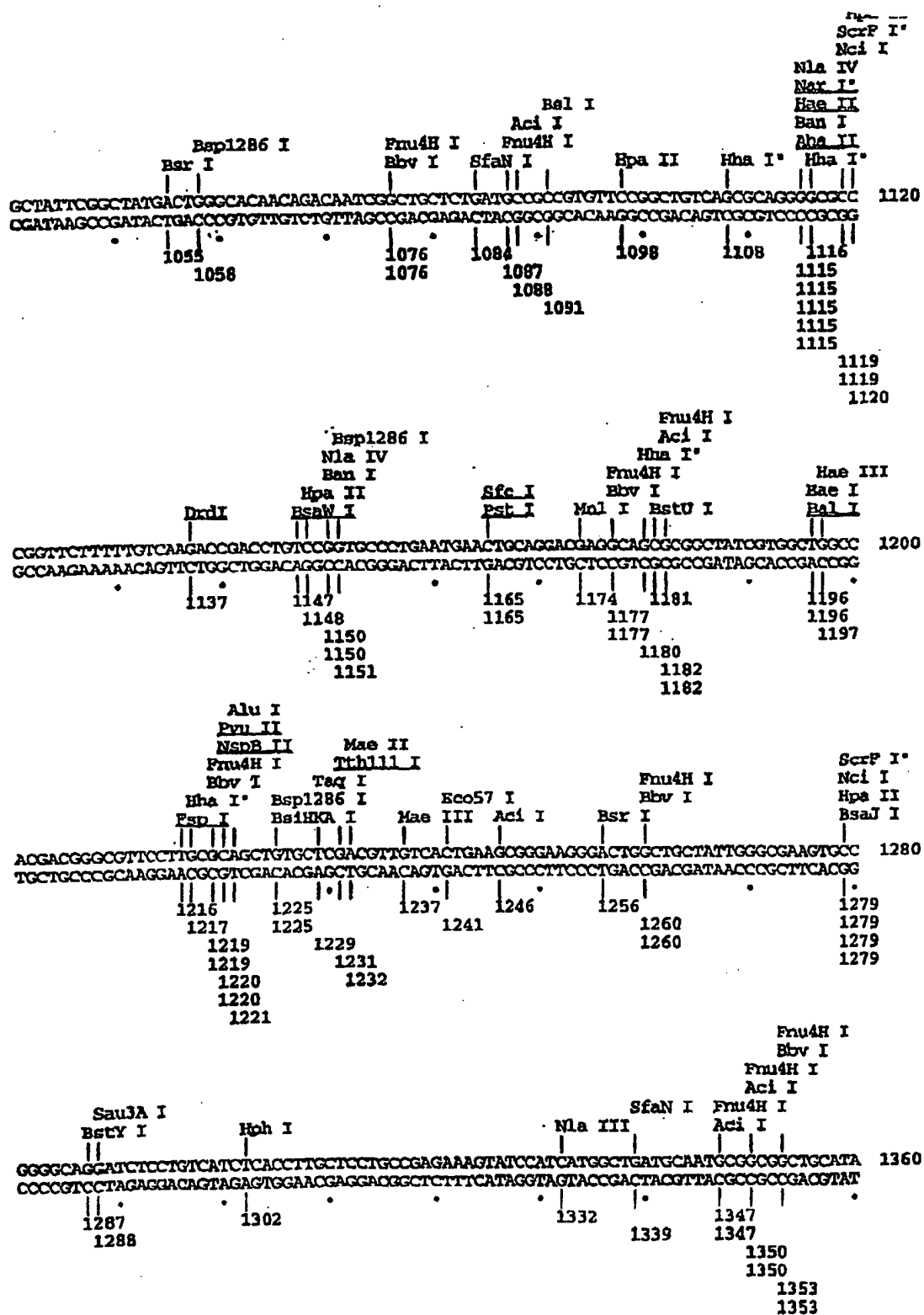


Fig 2(a) cont'd

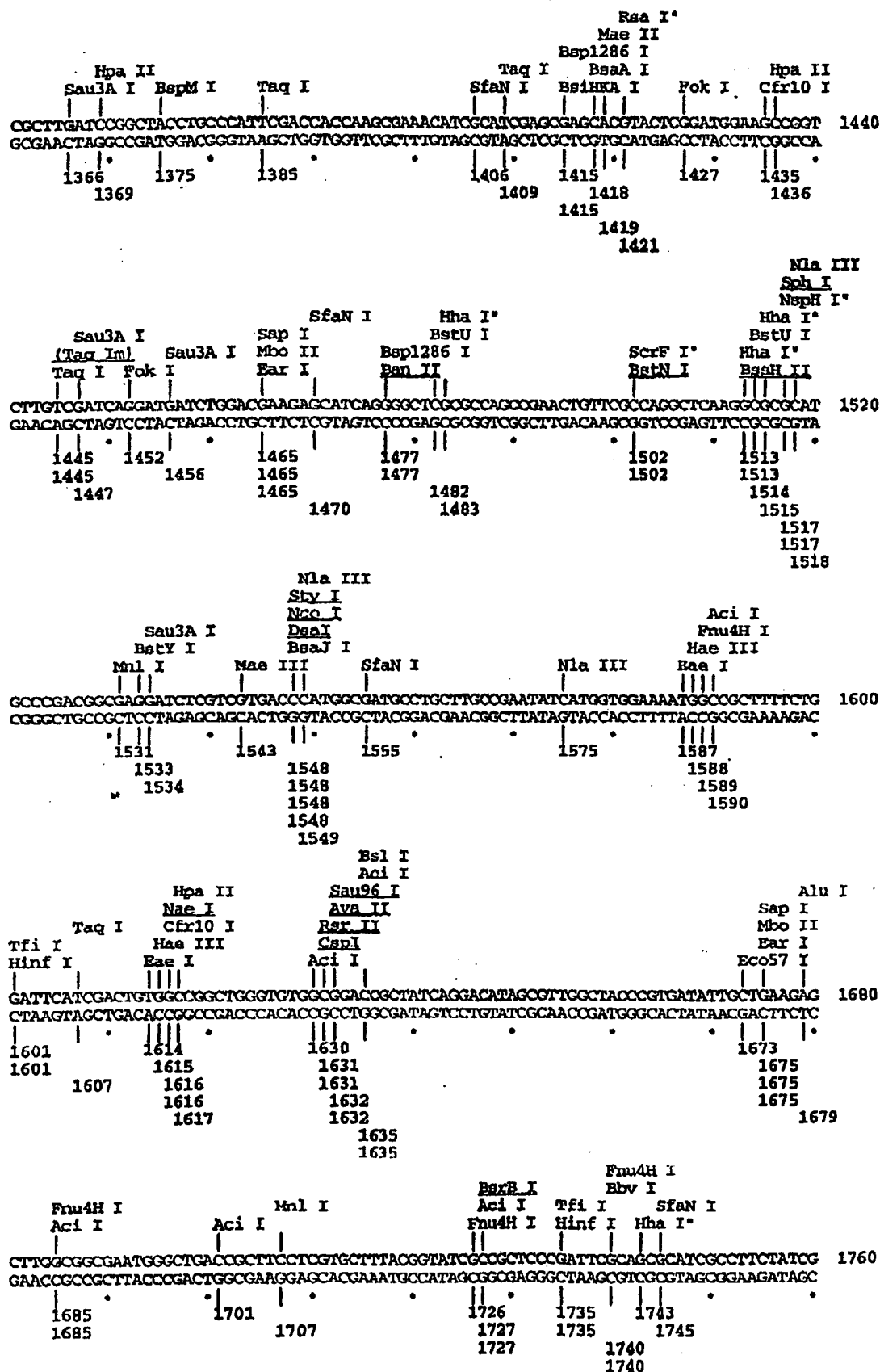


Fig 2(a), cont'd.

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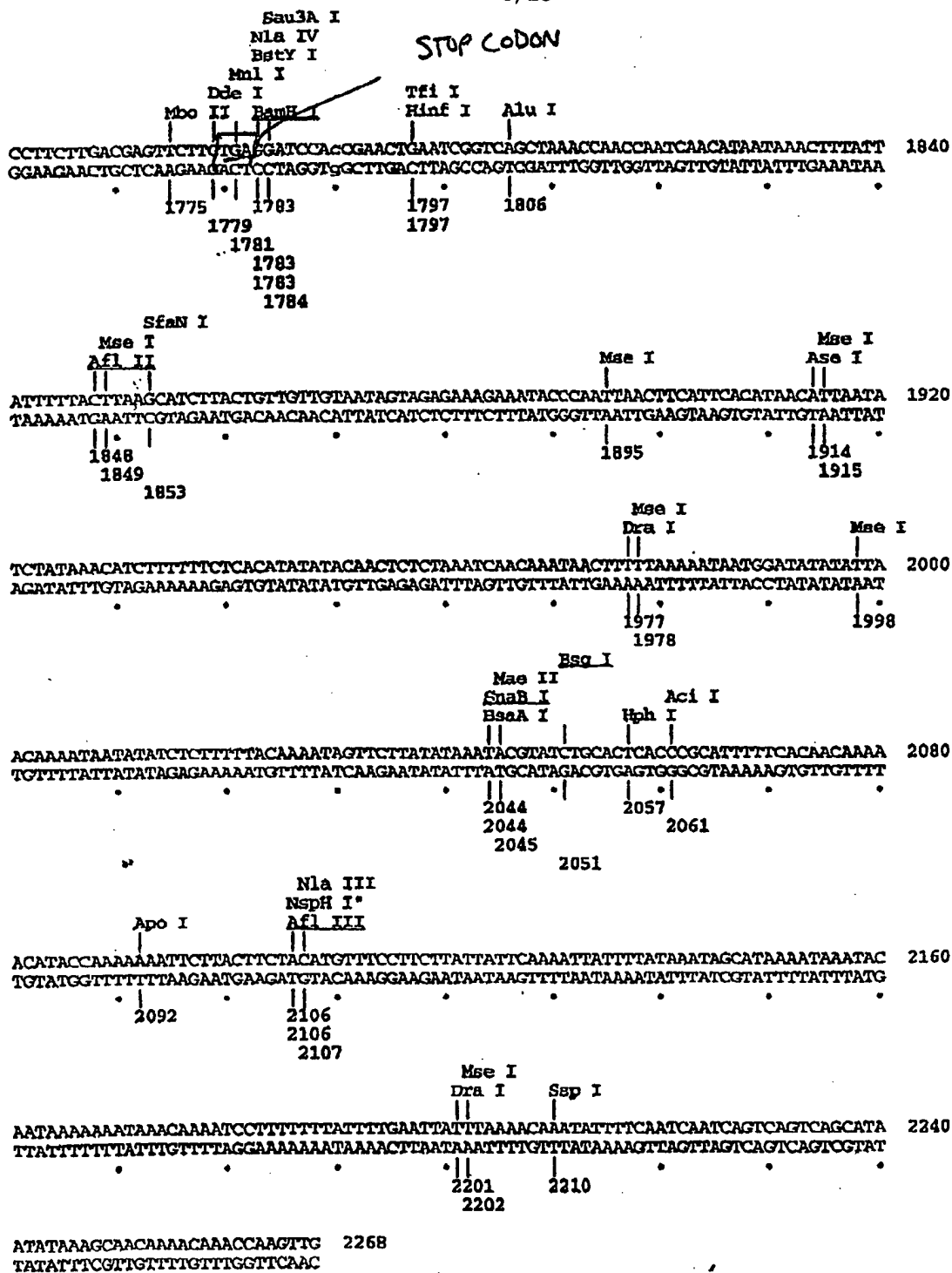


Fig 2(a), cont'd.

BICH3 -> Full Restriction Map

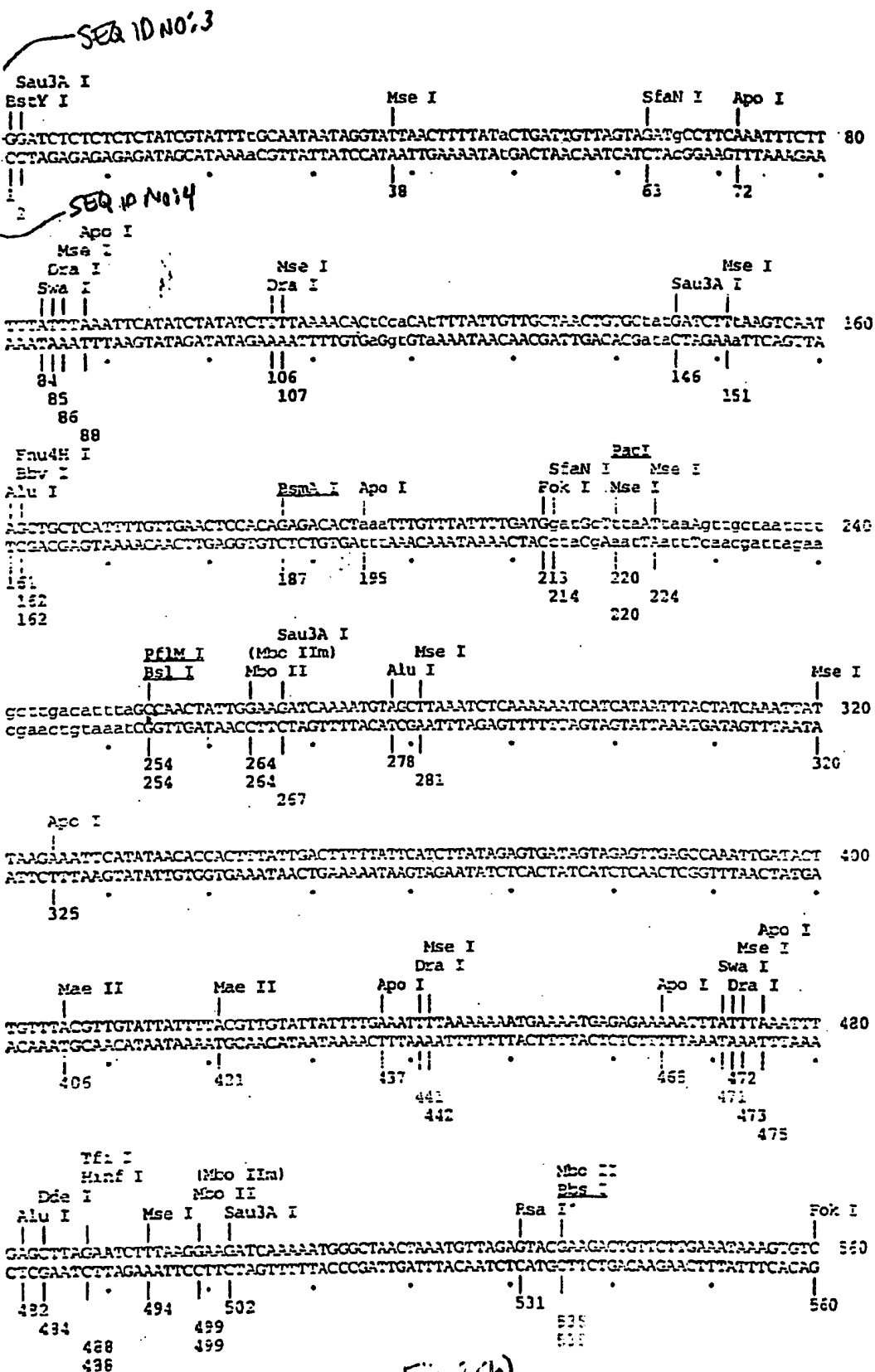


Fig. 2(b)

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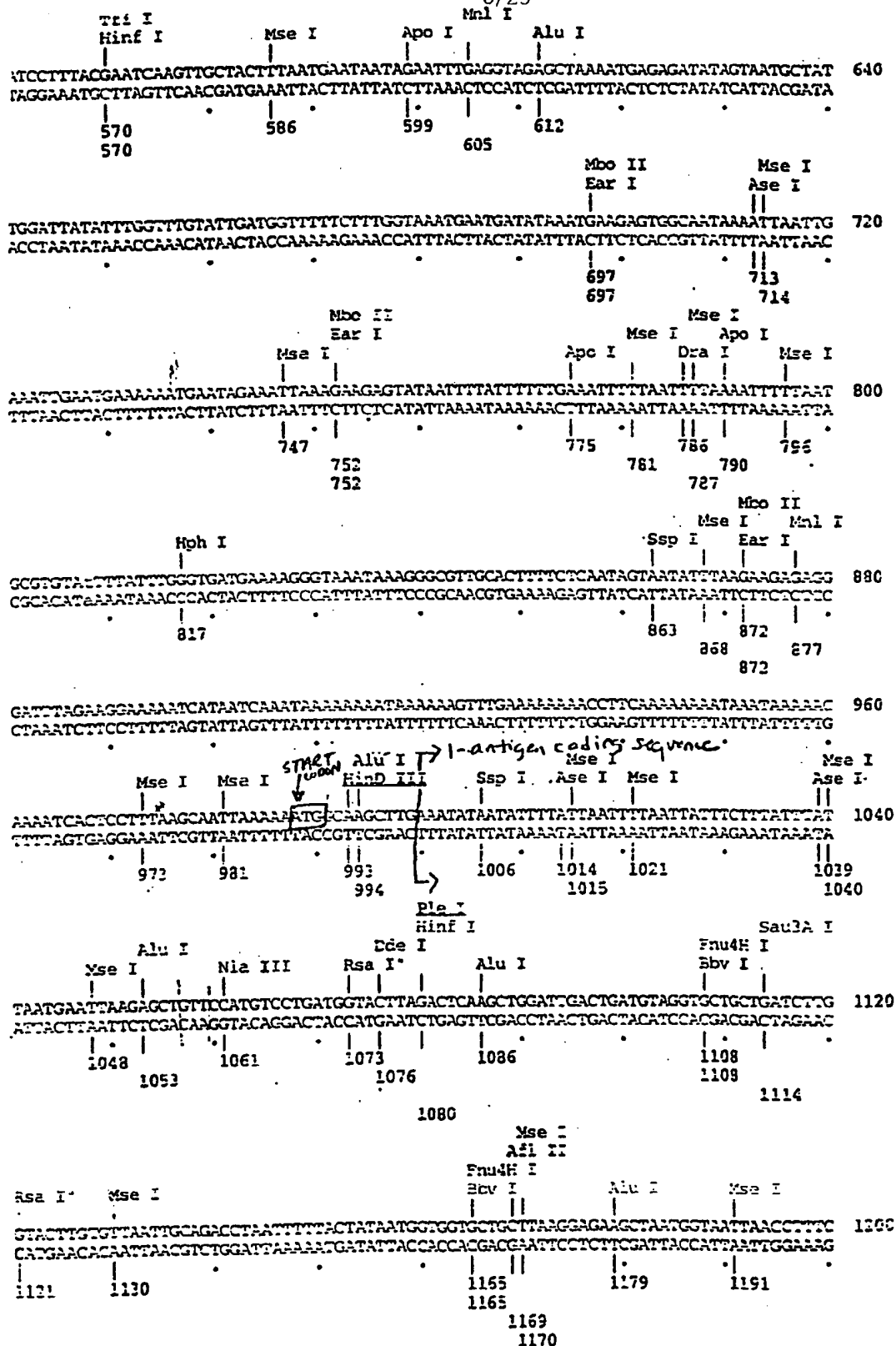
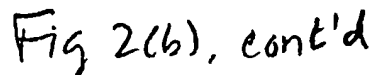


Fig 2(b), cont'd



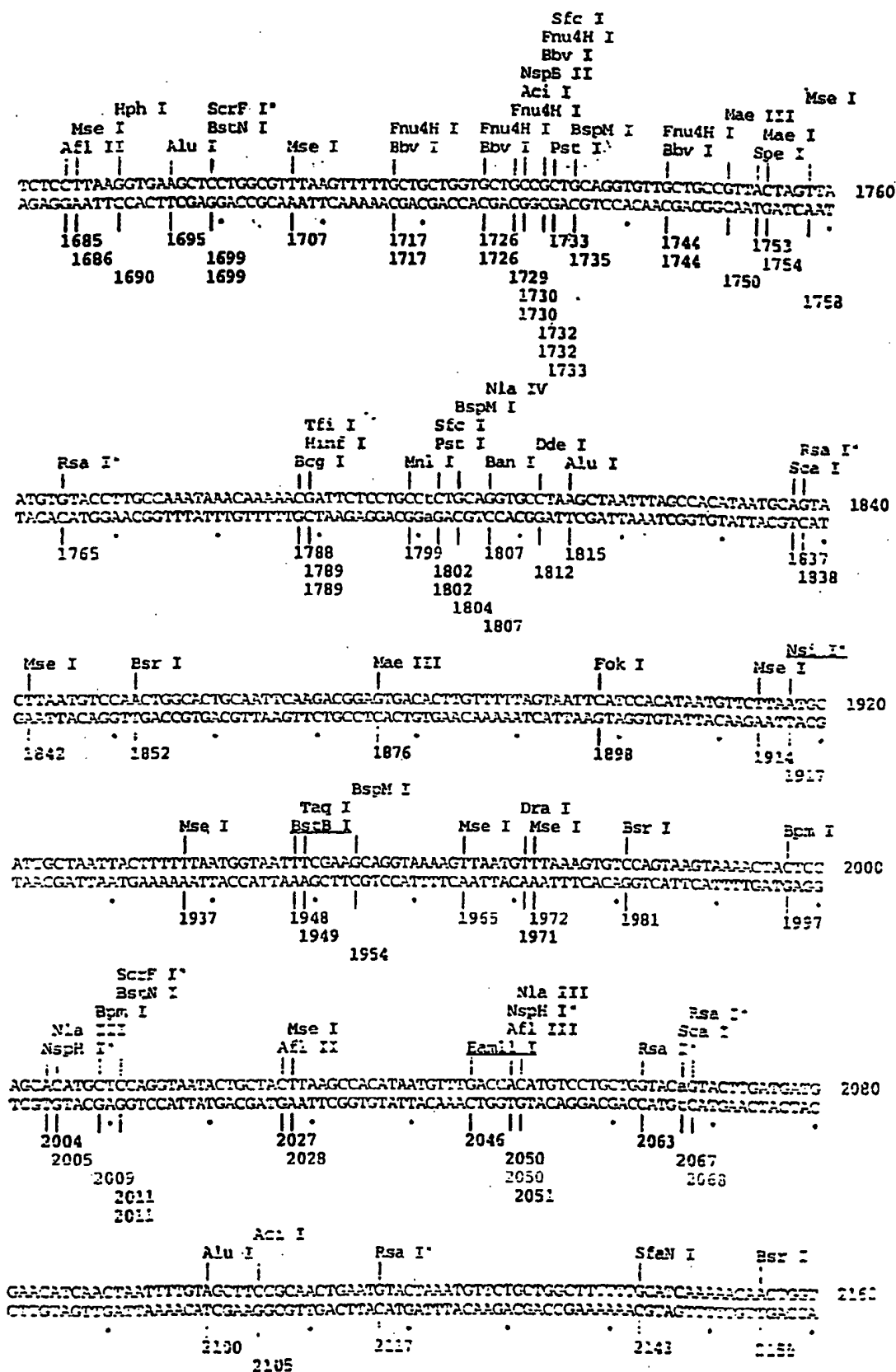


Fig 2(b), cont'd

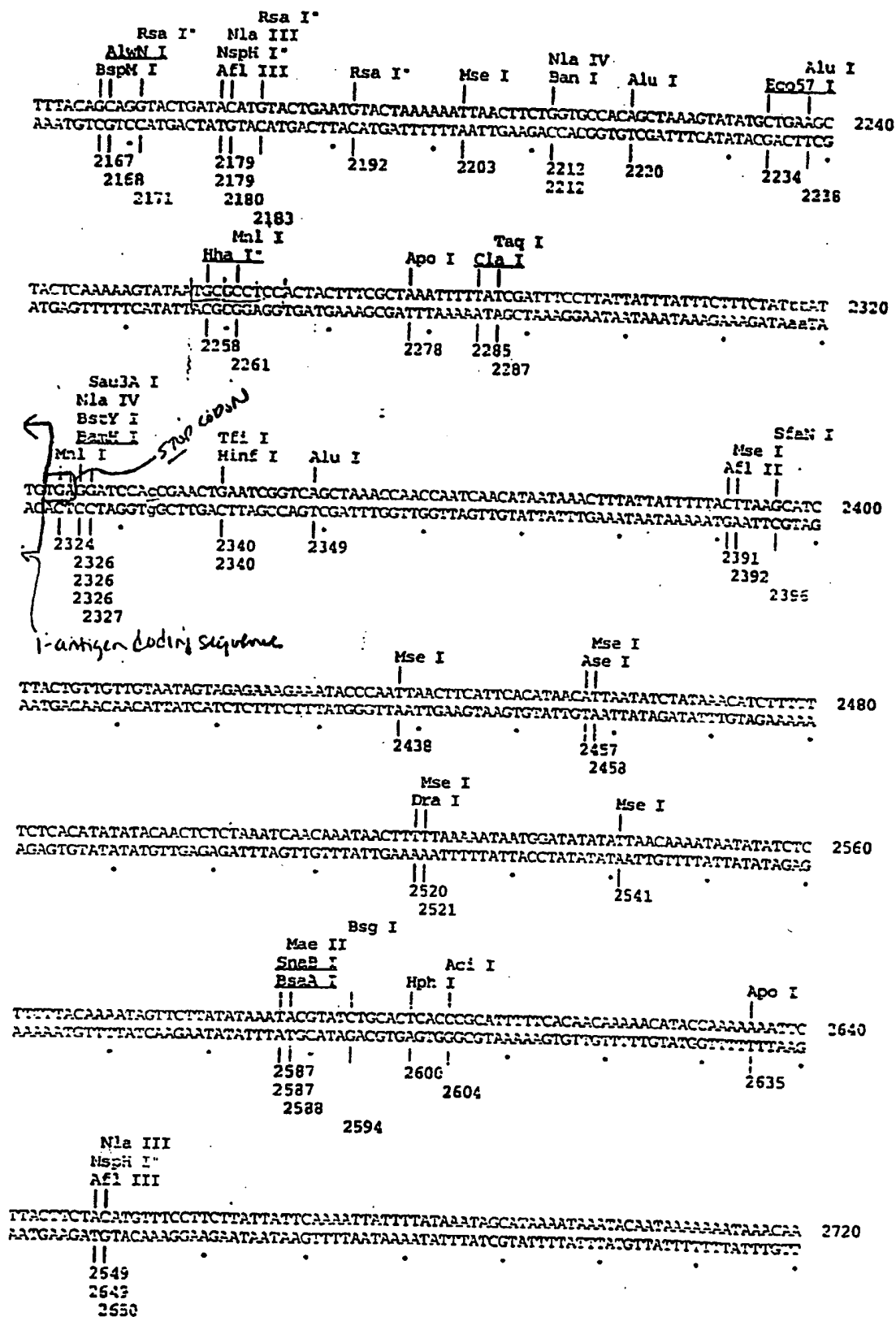


Fig 2(b), cont'd

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Mse I
Dra I Ssp I
||
AATCCTTTTATTTTGAATTATTTAAACAAATATTTCAATCAATCAGTCAGTCAGCATAATATAAGCAACAAAAC 2800
TTAGGAAAAAATAAACTTAATAAATTTGTTTATAAAAGTTAGTTAGTCAGTCAGTCGTATTATATTTGTTGTTTG
||
2744 2753
2745
AAACCAAGTTG 2811
TTTGGTTCAAC
.

Fig 2 (b), cont'd

Seq ID no: 5 G1
Seq ID no: 6 G5

1 MKNNILVILLISLFINELRAVPCPDGTQ-QAG-LFDVGAADLGTCVNCRPNFYV
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55 --GGAA-----QGEANGNP
59 FVPGASTCPCPKKQDAGAPNPATANLVTCNVKCPAGTALAGGNTDYAAILTECUNC
68 ---P-----AAN-NAARGICVPCQINRVGSVTNAGDLATLATQCTGTGTALDDGVTDV
119 RINFYNENAPNFENAGASTCTACPVNRVGGALTAGNATVACQCNVACPTGTALDDGVTDV
120 FDRSAAQCVKCKPNFYNGSPQGEAFGVQVFAAGAAAGVAAVTSQCVPCOLNK--NDS
179 YVRSFTECVKCRINFYNGN--GNTP-----FHPGX-----SQCTPCPAIKPANVA
178 PATAGAQANLATQCSNQCTGTVLDDGVTLVFNNTSALCVKCRPNFYNGGSPQGEAFGV
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325 -YVILQTECLNCAANFYFDGNNFQAGSSRCKACPANKVQGAATAGGTATTLAQCALFCF
355 XGTVLDDGTTNFVASATECTKCSAGFFASKTGTGTAGTDTCTECTKKLTSGAPAKVYAE
384 AGTVLDDGTTSTYKQAASECVKCRANFYTTKQTDWVAGIDICTSCNKKLTSGAEANLPES
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Fig. 3(a)

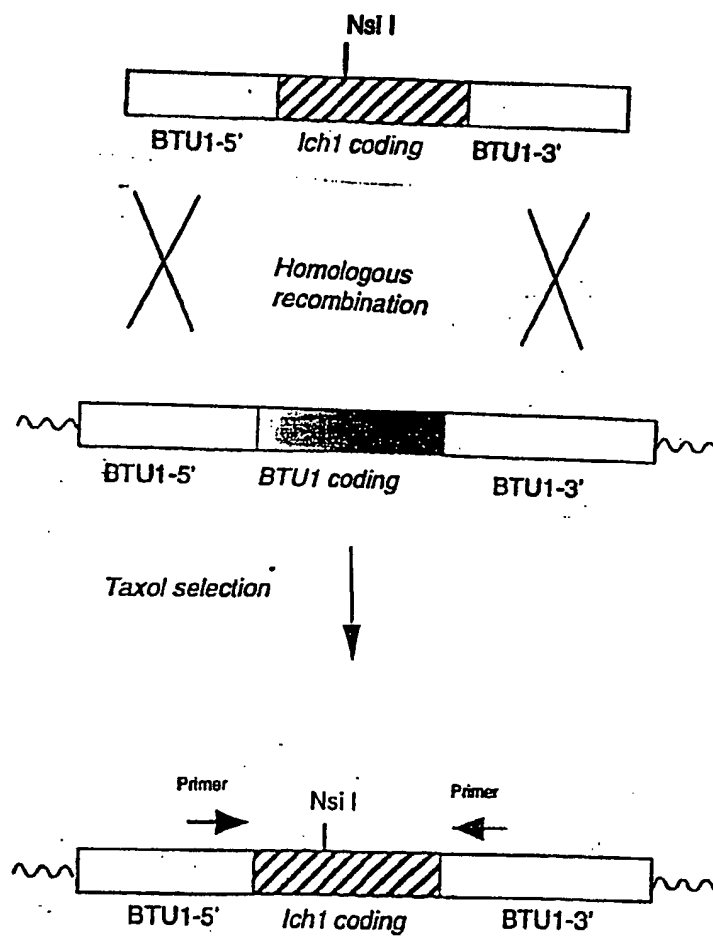


Fig. 4

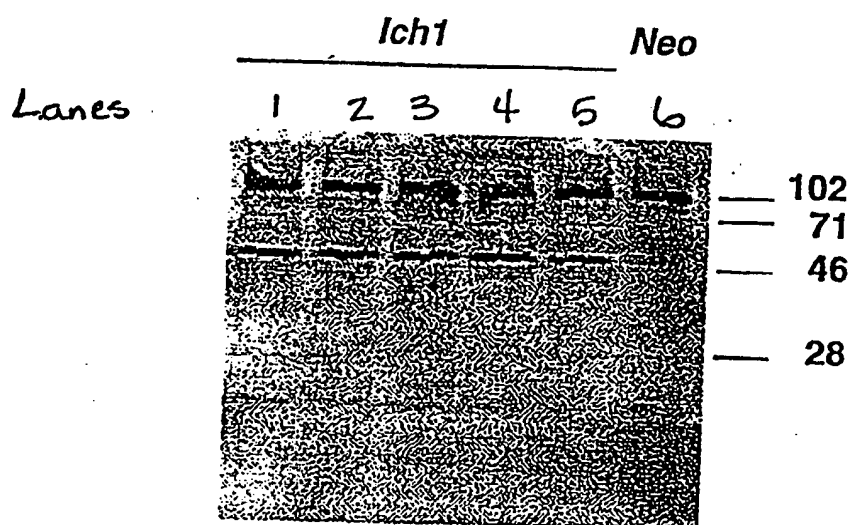
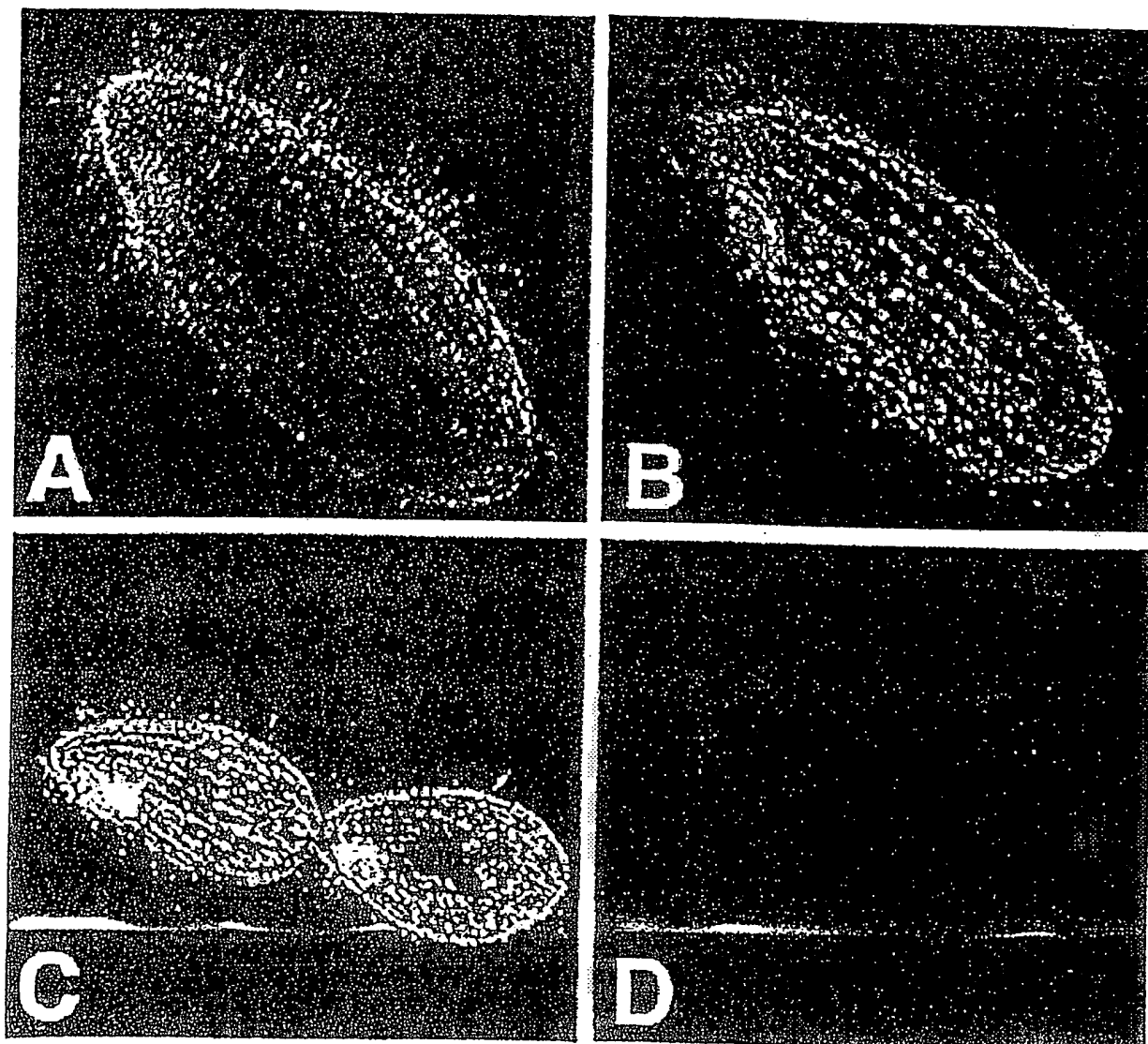


FIGURE 5

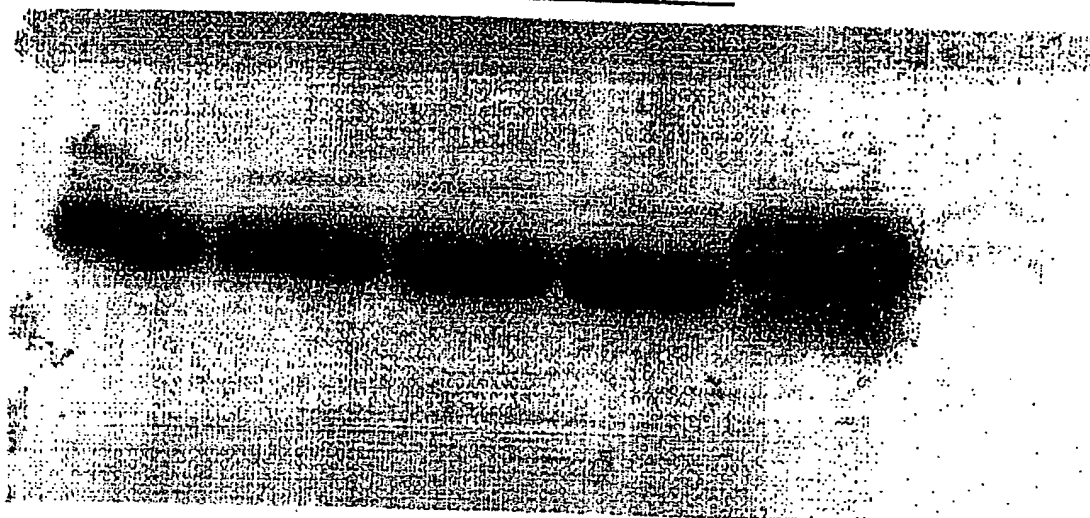


FIGURES 6A-6D

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(a) total protein from cell pellet

-gpi/G1 (50/69) G1 neo



(b) medium without cells

-gpi/G1 (50/69) G1 neo

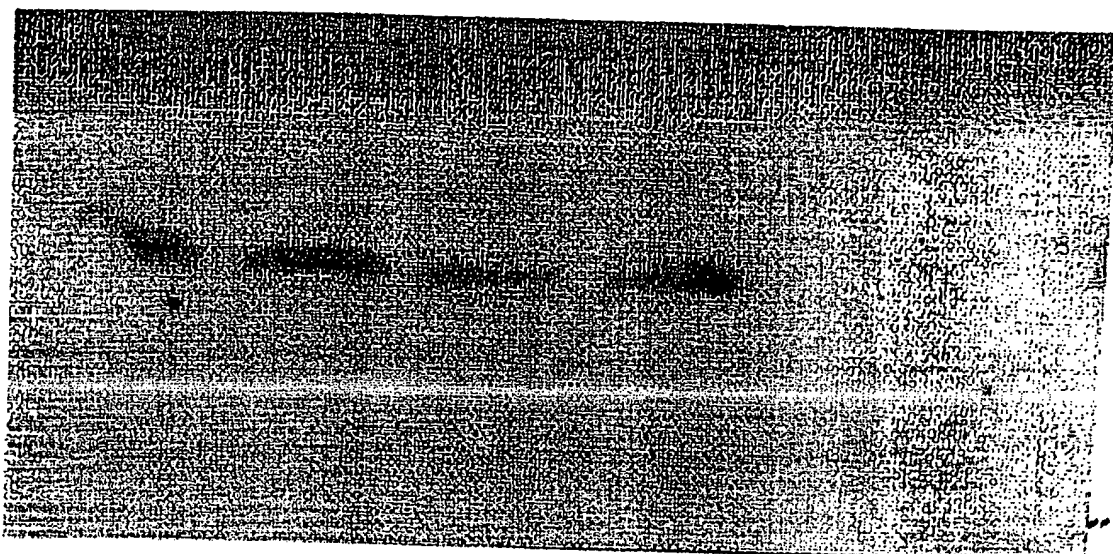


Fig. 7

Tryp./OVA Ich/OVA neo



Fig. 8

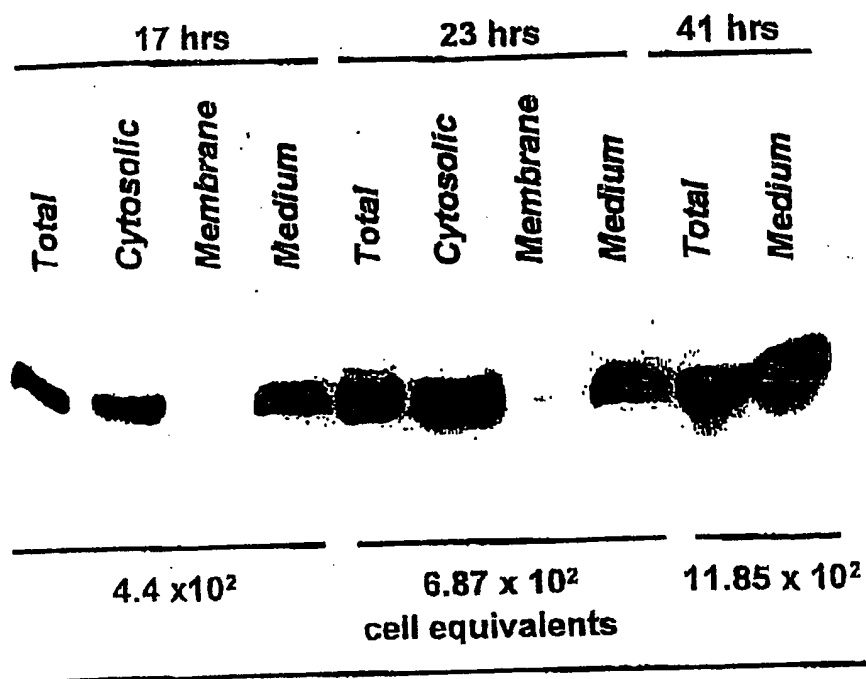


Fig. 9

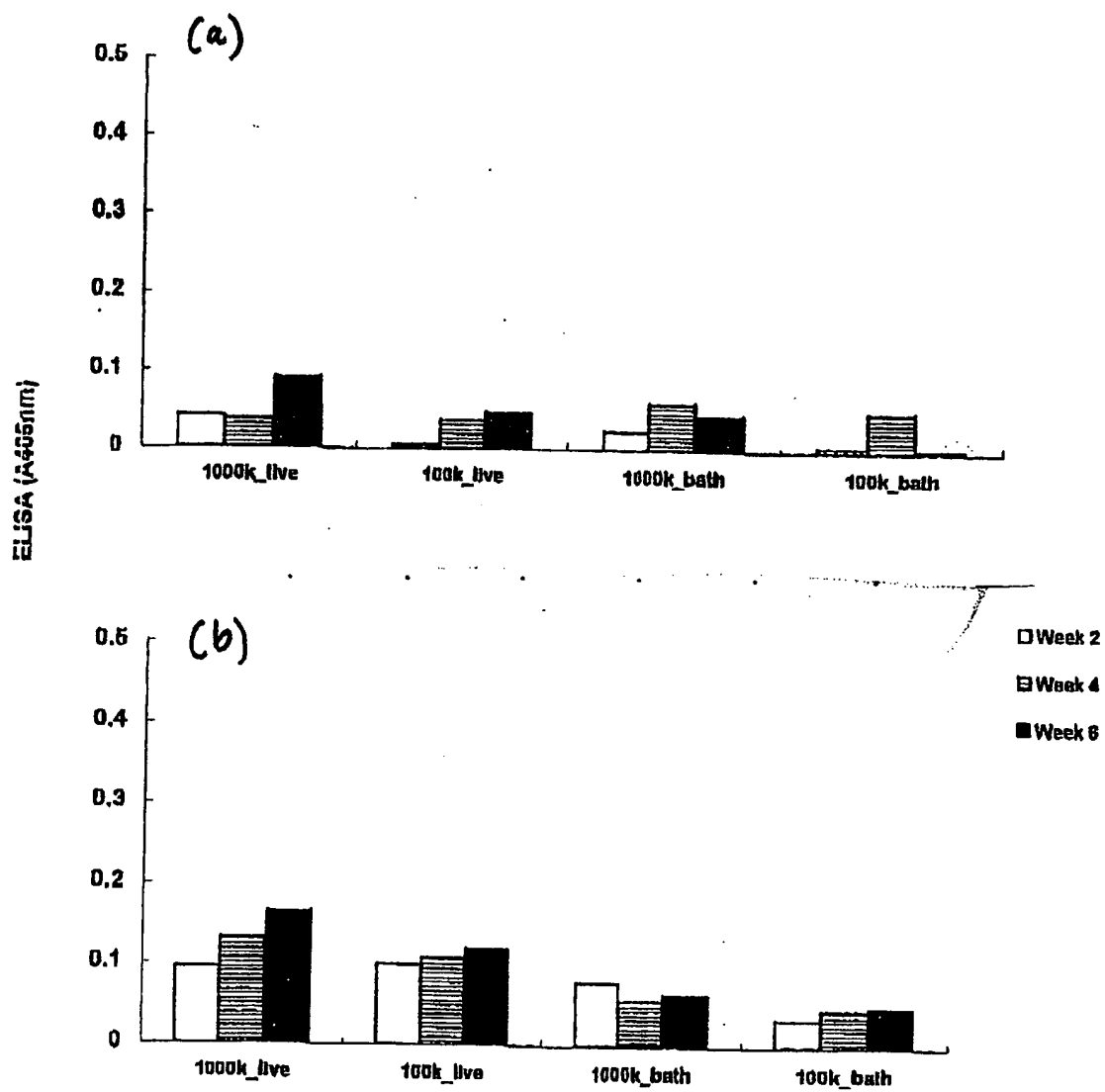
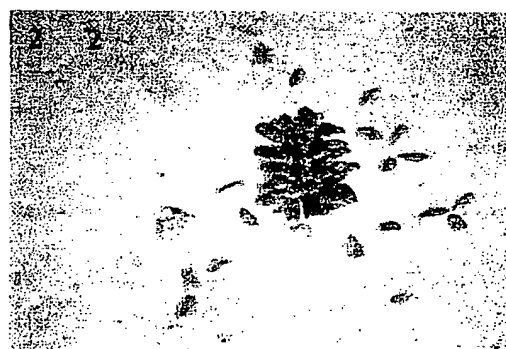


Fig. 10



Serum: anti-live Tneo (1 : 20)
(negative control)



Serum: anti-live TG1 (1 : 20)

Fig. 11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02966

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/79 C12N15/85 C12P21/00 G01N33/569 A61K39/002
A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GAERTIG J ET AL: "Electroporation-mediated replacement of a positively and negatively selectable β -tubulin gene in <i>Tetrahymena thermophila</i> " PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, May 1994 (1994-05), pages 4549-4553, XP002141863 WASHINGTON US the whole document --- -/--	1,3-5

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

6 July 2000

Date of mailing of the international search report

25/07/2000

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NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/02966

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLARK TG ET AL: "Developmental expression of surface antigen genes in the parasitic ciliate Ichtyophthirius multifiliis" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, July 1992 (1992-07), pages 6363-6367, XP002098111 WASHINGTON US the whole document	2,6-16, 41-52
A	GAERTIG ET AL: "High frequency vector-mediated transformation and gene replacement in Tetrahymena" NUCLEIC ACIDS RESEARCH, GB, OXFORD UNIVERSITY PRESS, SURREY, vol. 22, no. 24, 1994, pages 5391-5398-8, XP002128557 ISSN: 0305-1048 cited in the application the whole document	1-6
A	KAHN R W ET AL: "TRANSFORMATION OF TETRAHYMENA THERMOPHILA BY MICROINJECTION OF A FOREIGN GENE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 90, no. 20, 15 October 1993 (1993-10-15), pages 9295-9299, XP002044342 ISSN: 0027-8424 cited in the application the whole document	1-6
P, X	GAERTIG J: "Surface display of a parasite antigen in the ciliate Tetrahymena thermophila" NATURE BIOTECHNOLOGY, vol. 17, May 1999 (1999-05), pages 462-465, XP002141865 NATURE PUBLISHING., US ISSN: 1087-0156 the whole document	1-52